# The effect of UV-B radiation on flavonoid accumulation and gene expression in *Marchantia polymorpha*

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by

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Abstract of a Thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Biochemistry.

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William Armstrong Clayton

*Marchantia polymorpha* is the closest living representation of the earliest land plants and as such may give insight into the response that allow the first land plants to transition from the aquatic environment onto the terrestrial. One such adaption allowing this transition was to that of UV-B radiation and is the main focus of this study.This thesis investigates the effect of UV-B radiation on the basic land plant *M. polymorpha* and its responce through the production of secondary metabolites, in particular flavonoids. How flavonoid compounds may protect *M.* polymorpha and the gene regulation that contributes towards stress related defense and secondary compound production is also investigated.

*M. polymorpha* plants were subject to UV-B fluence at two seperate levels to stimulate both an acclimation and stress related response. Plants were then measured for the production of UV-B absorbing compounds, in particular the flavonoids, and also the localisation in thallus tissue of such compounds. The genetic response was also analysed using a combination of RNA-seq and nCounter techniques. Specific mutant plants with altered flavonoid amounts were also analysed to help further determine the genetic interactions that contribute to protection.

*M. polymorpha* was found to respond to UV-B through enhanced levels of UV-B absorbing compounds and in particular flavonoids, of which the flavones were the most predominant. Apigenin-based flavones were found in the highest amounts followed by luteolin-based

flavones. The ratio of Apigenin to Luteolin-based flavones was seen to shift towards luteolin in high UV-B responses. Apigenin was suggested to be required for UV-B screening functions under low fluence while high fluence treatments, that induce ROS may require Luteolin-based flavones that are able to scavenge ROS more effectively. Under low fluence conditions flavone compounds accumulated strongly in the epidermal layers suggesting a strong UV-B screening function.

RNA-seq revealed that *M. polymorpha* responds to UV-B through the UVR8 pathway similar to that of higher plants. We determined the UVR8 pathway in *M. polymorpha* contains the main regulators COP1, HY5 and RUP1 and act to enhance flavonoid biosynthetic genes such as CHS, CHI and FNS. RNA-seq and nCounter analysis also revealed differential expression in stress related genes that may offer protection independently of the UVR8 pathway and flavonoids. Mutant lines with altered flavonoid amounts were also tested under UV-B irradiance and showed that lower flavones concentrations resulted in enhanced damage to plant thallus. Elevated flavones resulted in enhanced protection and a reduction in thallus damage and loss of flavones altogether resulted in severe stunting in plants grown under UV-B.

Overall we determined that *M. polymorpha* responds through UVR8 for the production of flavone compounds and this production results in protection against the deleterous effects of UV-B. Such adaptive mechanisms likely assisted early land plants to transition from aquatic to land environments by reducing UV-B burden.

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# Abbreviations

| | |
|---|---|
| ˚C | degree centigrade |
| 35s | 35s promoter, from the cauliflower mosaic virus |
| 4CL | 4-coumarate:CoA ligase |
| $Ab_{XXXnm}$ | absorbance at the wavelength indicated by XXX in nanometres |
| bHLH | basic helix-loop-helix domain protein |
| bp | base pair |
| C4H | cinnamate 4-hydroxylase |
| cDNA | complementary deoxyribonucleic acid |
| CE | controlled environment |
| CHI | chalcone isomerase |
| CHS | chalcone synthase |
| Chl | chlorophyll |
| COP1 | constitutively photomorphogenic 1 |
| COX | cytochrome c oxidase |
| Ct | value crossing threshold value |
| DNA | deoxyribonucleic acid |
| DW | dry-weight |
| ERF | ethylene response factor |
| F3H | flavanone 3b-hydroxylase |
| FLS | flavonol synthase |
| FNR | flavanone 4-reductase |
| FNS | flavone synthase |
| g, kg | gram, kilogram |
| GL3 | glabra 3 |
| h | hour |
| HPLC | high performance liquid chromatography |
| HSP | heat shock protein |
| HY5 | elongated hypocotyl 5 |
| Hz | hertz |
| LC-MS | liquid chromatography - mass spectrometry |
| MAPK | mitogen activated protein kinase |

| | |
|---|---|
| min | minute |
| µL | microlitre |
| µM | micromolar |
| mL | millilitre |
| mM | millimolar |
| mRNA | messenger ribonucleic acid |
| MS | Murashige and Skoog: tissue culture medium |
| MYB | class of transcription factor |
| nm | nanometre |
| PAL | phenylalanine ammonia-lyase |
| PAR | photosynthetically active radiation |
| PCR | polymerase chain reaction |
| PR | pathogen-related |
| PSII | photosystem II |
| qRT-PCR | real time quantitative polymerase chain reaction |
| RNA | ribonucleic acid |
| RNS | reactive nitrogen species |
| ROS | Reactive Oxygen Species |
| rpm | revolutions per minute |
| RT | retention time |
| RUP1 | repressor of photomorphogenesis 1 |
| sec | second |
| SPA1 | suppressor of *phyA-105* |
| SUB1 | short under blue light 1 |
| TBE | Tris-Borate-EDTA |
| TF(s) | transcription factor(s) |
| Tm | temperature |
| UV | ultraviolet |
| UV-B | ultraviolet radiation between 280-320 nm |
| UVR8 | uv resistance locus 8 |
| v/v | volume per volume |
| w/v | weight per volume |
| WT | wild type |

| | |
|---|---|
| WD40 | WD40-repeat protein |
| µg/g | µg per gram |
| UVI | UV index |

# Chapter 1: Introduction

## 1.1 Evolution of land plants

The ancestor of modern plants (embryophytes) colonised the terrestrial habitat of earth 450-500 million years ago and this was a key step in the evolution of life on earth as we know it (Sanderson *et al.*, 2004, Wellman, 2010, Wellman *et al.*, 2003, Bateman *et al.*, 1998). To the present time, three major plant groups have evolved: Bryophytes (liverworts, hornworts and mosses), pteridophytes (lycophytes and monilophytes) and the spermatophytes (seed plants) that dominate most environments.

Present day land plants are widely accepted to have evolved from a green algae ancestor, in particular the Streptophyte algae (charophycean algae) of which the Zygnematales have been proposed as the closest relatives to land plants today (Springer & Gatesy, 2014, Zhong *et al.*, 2013, Wodniok *et al.*, 2011). Streptohyte algae were largely adapted to fresh water environments, which may have played a key role in their early adaption to land. Proposed as the first algae to exploit fresh water environments, streptophytes may have been able to adapt towards more moist environments in the proximity of water, and later towards dry areas dependent on freshwater rainfall to survive (Becker & Marin, 2009). The first step onto land may have also been mediated by a symbiotic relationship with fungi and a photosynthetic organism, most likely a charophycean algae (Heckman *et al.*, 2001, Selosse & Le Tacon, 1998). Such symbiotic relationships may have provided protection and helped early plants adapt to the harsh conditions faced on land. The proposed first land plant to have made this step is thought to be most closely related to the extant liverworts (Qiu *et al.*, 1998, Wellman et al., 2003)

## 1.2 Adaption to stress on land

The first land plants faced many new challenges in their attempts to colonise the early land environment, encountering many problems including desiccation, UV stress, large temperature fluctuation and osmoregulation (Becker & Marin, 2009, Waters, 2003, Graham *et al.*, 2000, Floyd & Bowman, 2007, Rensing *et al.*, 2008). In order to colonise the land they needed to overcome the challenges that these problems presented.

### 1.2.1 Desiccation tolerance

Early land plants survival depended on tolerance and avoidance of desiccation in order to survive the relatively dry climate on land. Early plants had simply architecture and lacked the morphological or physiological strategies to prevent water loss. In their transfer from aquatic habitats to land, early plants would need to evolve the mechanisms to negate water loss from cells where exposure to air in the absence of surrounding water would likely quickly dry and kill them (Waters, 2003, Oliver *et al.*, 2005).

Vegetative desiccation tolerance is common within the bryophytes but rare within the extant vascular plants that have the ability to transport water (Alpert, 2000). The bryophytes are the closest living relatives of early land plants and the desiccation tolerance mechanisms present in them may give insight into how the first land plants were able to tolerate desiccation and thus colonise land (Oliver et al., 2005, Rensing et al., 2008).

In order for desiccation tolerance to be effective the ability to limit damage from desiccation and rehydration whilst maintaining cellular integrity the ability to activate repair or mobilise repair mechanisms upon rehydration must be present (Bewley, 1979). In order to achieve this extant plants have evolved a variety of strategies. However, focus on the mechanisms of protection adopted by bryophytes may give greater insight into the strategies used by early plants.

Desiccation tolerance of bryophytes utilises constitutive protection of cellular structures with a rehydration induced repair and recovery process (Oliver *et al.*, 2000, Oliver et al.,

2005). Cellular ultrastructure and membranes do not seem to be damaged during the desiccation process (Platt *et al.*, 1994), thought to be protected via the constitutive expression of the Late Embryogenesis Abundant (LEA) proteins, abscisic acid (ABA) and accumulation of soluble sugars (Oliver et al., 2005, Rensing et al., 2008). Once desiccation occurs the cells are largely intact ready for a rehydration event. Rehydration however, can itself cause large-scale cellular damage (Osborne *et al.*, 2002) and an effective recovery and repair process must be utilised. On rehydration the bryophyte's gene expression patterns change in favour of transcripts that encode rehydrins, proteins expressed in response to rehydration to help mediate the repair and recovery process. Photosynthesis rapidly recovers after rehydration (10-20min) and the chloroplast repair and recovery may not be dependent on new protein synthesis. This indicates photosynthetic machinery is well protected and able to quickly supply energy to repair other cellular damage in rehydration events (Oliver et al., 2005).

### 1.2.2 Thermal tolerance

The earliest land plants evolved from relatively temperature stable aquatic environments to that of land where temperatures can be extreme and fluctuate daily. Protection of the photosynthetic machinery would have been of particular importance to early plants evolving to live in these environments. Isoprene has been shown to confer thermal tolerance in plants through its addition to the phospholipid membrane giving greater stability at higher temperatures (Siwko *et al.*, 2007, Sharkey & Yeh, 2001). However isoprene emission is found to be lacking in liverworts and hornworts but present in mosses and ferns (Hanson *et al.*, 1999). The relatively low growth form of liverworts and hornworts is suggested to protect against thermal stress compared to the largely aerial mosses, which would have incurred much more dramatic temperature fluctuations (Hanson et al., 1999, Waters, 2003).

Thermal tolerance is also mediated through the more conserved heat shock response pathway that is present in both bacteria and eukaryotes. The heat shock response pathway is activated in response to high temperature stress and mediates the expression of heat shock proteins (HSPs). HSPs are largely chaperone proteins that assist protein folding and

prevent protein aggregation within the cell (Lindquist & Craig, 1988, Craig *et al.*, 1994). The HSP family of small heat shock proteins (sHSPs) are particularly important and form a large and diverse group within plants, but are also found in a large array of organisms (Waters, 2013). Present in algae as only a cytosolic group, the sHSPs appear to have undergone a burst of duplications associated with the bryophyte *Physcomitrella*, and later duplication associated with the divergence of *Selaginella*, a lycophyte (Waters, 2013). The subsequent evolution, specialisation or adaption of these genes quite likely helped the early land plants to adapt to thermal tolerance in order to colonise the land.

### 1.2.3 Adaption to UV

Early Earth harboured little atmospheric oxygen or ozone and subsequently received very high doses of damaging UV-C (<280 nm) and UV-B (280-320 nm) radiation (Walker, 1976). Such high dosage prevented any growth on land and only organisms shielded by the water column were able to survive. The gradual release of oxygen from aquatic photosynthetic cyanobacteria and later eukaryotic algae reduced atmospheric $CO_2$ and allowed $O_2$ levels to climb (Walker, 1976). As atmospheric $O_2$ levels rose the amount of UV-C and UV-B protective ozone ($O_3$) also increased allowing for terrestrial colonisation by the first land plants (Rozema *et al.*, 1997, Caldwell, 1979). The levels of $O_3$ were lower than present day however, and the incident UV radiation experienced by these early land plants was much higher (Walker, 1976, Caldwell, 1979). UV-B radiation results in damage through its absorption by the purine and pyrimidine bases that make up DNA, membranes, photosynthetic machinery and phytohormones, of which DNA damage is thought to be most significant (Rozema et al., 1997). In response plants have developed many strategies to deal with this damage including DNA repair mechanisms, ROS scavengers, scattering or reflection of UV light and UV absorbing compounds (Fig. 1.1) (Rozema et al., 1997, Jordan, 2002).

The UV absorbing compounds are of particular interest in the evolution of land plants. The flavonoid compounds, known to be involved in UV protection, are absent from proposed green algae ancestors and present in bryophytes, representing the extant relatives of the first land plants (Waters, 2003). This acquisition of flavonoid synthesis may have been a key step facilitating the colonisation of land and protection from UV light. Flavonoid compounds

are of particular interest as they form part of a group of protective pigments that are able to absorb within the UV-B range (280-320nm) whilst still allowing light of higher wavelengths to pass through for photosynthesis. Sinapate esters are proposed to have greater protective properties than flavonoids and may be important pigments in higher plants for the attenuation of UV-B, but are not produced in early land plants (Sheahan, 1996).

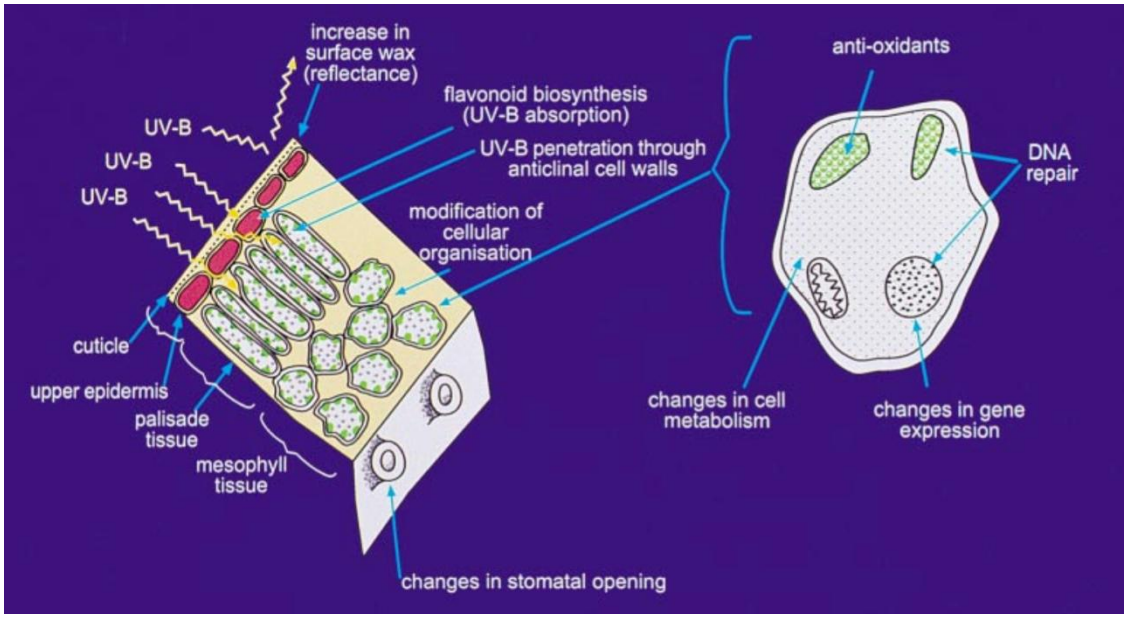



Figure 1.1: Protective mechanisms against UV-B. The schematic represents a cross-section of a leaf exposed to UV-B radiation, and a mesophyll cell illustrating cellular responses to UV-B (from Jordan 1996; Fig. 5).

## 1.3 UV induced damage in plants

### 1.3.1 DNA damage

DNA is particularly susceptible to UV damage which results mainly in the formation of cyclobutane pyrimidine dimers (CPDs), 6-4 photoproducts (6-4 PPs) and DNA double-strand breaks (DSBs) (Nawkar *et al.*, 2013, Jansen *et al.*, 1998). This mutation of DNA causes a disruption in cellular processes resulting from negative effects in DNA transcription and replication (Britt, 1996). DNA repair mechanisms to this damage include the plant-specific photoreactivation pathway, nucleotide excision repair (NER) pathway and homologous recombination (Liu *et al.*, 2000, Dubest *et al.*, 2002, Kimura *et al.*, 2004).

The photoreactivation pathway involves the photolyase enzyme that utilises light energy to repair DNA damage. Photolyase is able to repair both CPD's and (6-4) PPs and is present in many bacteria, fungi and plants yet non-functional in humans (Sinha & Häder, 2002). DNA photolyases transverse the genome and bind tightly to DNA on recognition of CPD's and (6-4) PPs. The photolyase contains a chromophore able to absorb the blue-light photon and transfer energy to the catalytic cofactor, most commonly a flavin–adenine dinucleotide ($FADH^-$). From here donation of an electron to the CPD mediates breakage of the cyclobutyl ring followed by electron transfer back to the flavin cofactor with restoration of the two original bases (Sinha & Häder, 2002, Sancar, 1996).

Nucleotide excision repair (NER) is also responsible for repair of DNA damage in plants but is independent of light. NER is able to recognise a wide range of DNA damages and induces several nicks on the damaged DNA strand to initiate its removal. These nicks occur either side of the recognised damage, which promotes excision of the damaged section by a DNA helicase. The gap produced is filled by DNA synthesis and joined by DNA ligase to restore the strand to its original undamaged state. In plants the main method of DNA repair from UV damage is through the photoreactivation pathway while only a small amount occurs through the light independent NER pathway (Britt, 1996, Sinha & Häder, 2002).

### 1.3.2 Damage to photosynthesis

Damage by UV-B to the photosynthetic machinery is mainly due to its effects on photosystem II (PSII) (Jansen et al., 1998, Takahashi & Badger, 2011). PSII is located in the thylakoid membrane and catalyses the transfer of electrons from water to plastoquinone, providing the electrons to enable photosynthesis to occur. Damage from UV-B to PSII occurs mainly through the degradation of the D1 and D2 proteins via UV-B sensitive sites within PSII (Fig. 1.2) (Jansen et al., 1998, Tyystjärvi, 2008). The degradation of D1 and D2 is highest at 300nm correlating with its high susceptibility to UV-B of 280-315nm (Jansen *et al.*, 1993, Jansen *et al.*, 1996). UV-B may also damage the manganese cluster in PSII resulting in

damage to the oxygen evolving complex and ultimately photoinhibition (Tyystjärvi, 2008). UV-B wavelengths are not the only damaging light to affect PSII as high levels of visible light also have an effect. The mechanisms of PSII damage and photoinhibition may be largely due to both UV and visible light in combination.

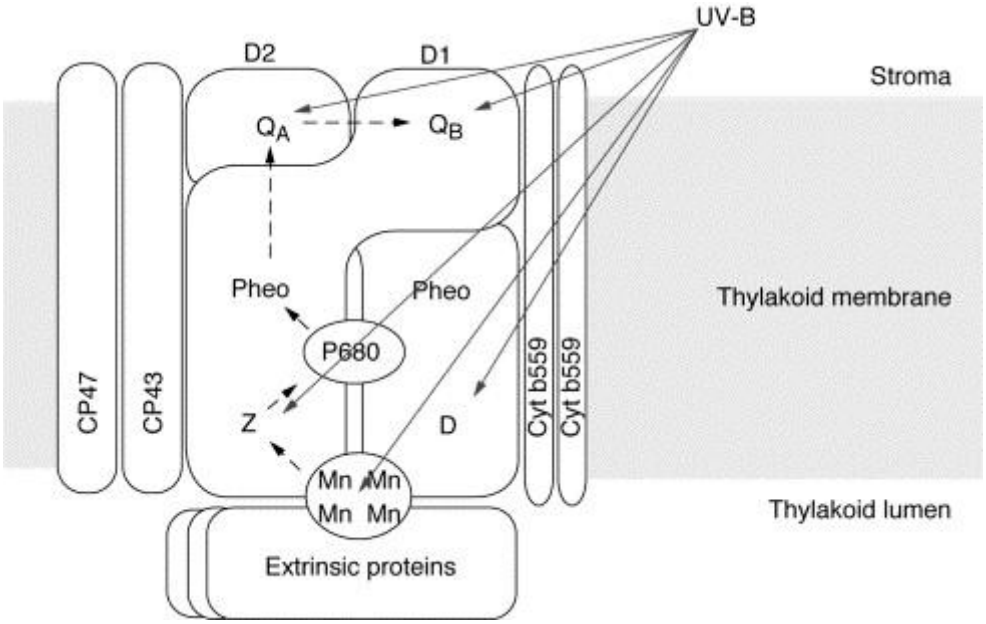



Figure 1.2: Schematic presentation of photosystem II (PSII), indicating photosensitizers proposed to be involved in its UV-B-mediated inactivation. P680 is the primary electron donor of PSII. Z and D are redox active tyrosines located on the D1 and D2 proteins, respectively; Z normally serves as the electron donor to P680. Electrons originate from water, the splitting of which is catalysed by a cluster of four manganese atoms. Extrinsic proteins are involved in stabilizing this reaction. On the acceptor site, a pheophytin (Pheo) serves as the primary electron acceptor. The plastoquinones, $Q_A$ and $Q_B$, are the secondary electron acceptors. Photosensitizers that absorb in the UV-B range and that have been proposed to play a role in PSII inactivation and/or D1-D2 degradation are marked by arrows (From Jansen et al. (1998)).

### 1.3.3 Reactive Oxygen Species

Reactive oxygen species (ROS) are chemically reactive species, often in the form of oxygen ions and peroxides. In plants ROS are an unavoidable by-product of essential processes such as photosynthesis and respiration. Their accumulation in plant cells can be both hindrance and help as they can be damaging to cellular processes yet are also implicated in cell signalling (Mittler *et al.*, 2004). ROS production in response to UV-B irradiation can be due to

the disruption of metabolic process such as photosynthesis or other factors such as increased activity of membrane localised NADPH-oxidase (Hideg *et al.*, 2013). Low level UV-B is able to generate ROS at levels that do not cause oxidative stress and may act as a signal for UV-B acclimation. Higher UV-B fluence causes much larger ROS production and a corresponding increase in damaging oxidative stress and plant cell death (Hideg et al., 2013, Kottuparambil *et al.*, 2012)

UV-B has the ability to not only increase hydrogen peroxide ($H_2O_2$) concentrations but also cleave hydrogen peroxide to produce hydroxyl radicals (Czégény *et al.*, 2014). These hydroxyl radicals are short lived and react at the site of their production. These hydroxyl radicals have been reported in chloroplast structures under high UV-B fluence yet limited detection methods restrict the reporting of such molecules under more realistic lower UV-B irradiance (Jordan, 2017).

The mechanism by which ROS are produced in plants in response to UV-B is broad and is likely not limited to one source (Jenkins, 2009). While the most likely source of ROS is due to disruption of the photosynthetic machinery (Barta *et al.*, 2004), NADPH oxidase activity was also stimulated by UV-B treatments in Arabidopsis (Mackerness *et al.*, 2001). $H_2O_2$ was found to have a direct effect on pathogen resistance gene response but not on the UV-B acclimation response through chalcone synthase (CHS) regulation. However nitric oxide (NO), another free radical produced in pathogen response by L-arginine in a reaction involving NO synthase (NOS), was found to increase in response to UV-B and the use of inhibitors of nitric oxide synthase (NOS) or NO scavengers resulted in decreased chalcone synthase activity, suggesting an important function of NO in UV-B tolerance during ROS burden (Mackerness et al., 2001).

It must be noted that ROS damage seen in UV-B irradiated plants may be limited to controlled environmental conditions that utilise high UV-B levels. ROS damage by realistic UV-B levels contained in ambient light may be much lower and act as a signal for normal acclimation response (Hideg et al., 2013, Hideg & Strid, 2017 "In Press")

## 1.4 UV response in plants

### 1.4.1 Specific UVR8 mediated UV-B response pathway in plants

Plants respond to the light environment around them through sensory photoreceptors in order to optimise growth or prevent potential damage. Plants contain blue light sensing cryptochromes, phototropins and Zeitlupe family members as well as the red / far red sensing phytochromes and also the UV-B photoreceptor UVR8 (UV Resistance Locus 8) (Rizzini *et al.*, 2011, Heijde & Ulm, 2012, Wu *et al.*, 2012). First identified in *Arabadopsis* mutants hypersensitive to UV-B (Kliebenstein *et al.*, 2002), UVR8 is required for UV-B responses to occur under UV-B conditions (Fig. 1.3) (Favory *et al.*, 2009, Heijde & Ulm, 2012). UVR8 is localised to both the cytoplasm and the nucleus, though upon UV-B irradiance, it is redistributed and accumulates rapidly within the nucleus (Kaiserli & Jenkins, 2007). This relocation to the nucleus is required for its function as it interacts with COP1 (CONSTITUTIVELY PHOTOMORPHOGENIC 1) and activates UV-B responsive genes including those involved in flavonoid biosynthesis, DNA repair and oxidative damage (Favory et al., 2009).

UVR8 is present *in planta* as an inactive homodimer which upon UV-B irradiance undergoes instant monomerisation for the formation of the active state after relocation to the nucleus with association of COP1 (Rizzini et al., 2011). Monomer formation is thought to occur through a proposed chromophore whereby UV-B absorbance by highly conserved tryptophan residues results in a structural change and monomer formation. In particular β-propeller subunits form a tryptophan dominated dimer interface which is held together by flanking arginine residues that form a complex salt bridge network. Photoreception disrupts salt bridges and triggers dimer dissociation allowing corresponding signal transduction (Christie *et al.*, 2012). Mutation of the key tryptophan residue Trp-285 to phenylalanine confers constitutive UVR8 dimerisation and loss of COP1 interaction, while mutation of Trp-285 to alanine produces constitutive monomer formation and constant COP1 interaction (Rizzini et al., 2011). Importantly computational analysis of the tryptophan residues and their interaction with surrounding arginine residues revealed an absorption maximum of 280-300 nm (Wu *et al.*, 2011). COP1 interaction also requires a 27 long amino acid sequence located towards the C-terminus and deletion of this region prevents COP1 binding and signal

transduction (Cloix *et al.*, 2012). UVR8 is also found to associate with chromatin through its ability to bind histones (Jenkins, 2014). What facilitates the binding and whether COP1 is required or interacts with chromatin bound UVR8 is yet to be determined (Jenkins, 2014). The perception of UV-B by a UVR8 chromophore and resulting monomerisation with interaction of COP1 is most likely the molecular mechanism leading to UV-B perception and UV-B signal transduction.

The plant response to UV-B involves a signal transduction pathway involving UVR8. Many of the major regulators of this pathway are currently unknown, yet COP1 is strongly involved as is the bZIP transcription factor HY5 (ELONGATED HYPOCOTYL 5). In light conditions HY5 is stabilised and acts as a positive regulator of photomorphogenisis, while in the dark COP1 in interaction with SPA (SUPPRESSOR OF *phyA-105*) acts as an E3 ubiquitin ligase complex targeting HY5 for destruction (Lau & Deng, 2012). This interplay between HY5 and COP1-SPA is a key regulator in the UV-B signal transduction pathway. COP1-SPA interaction with UVR8 in response to UV-B may inactivate its E3 ubiquitin ligase activity resulting in accumulation of HY5 in addition to UVR8-COP1 stimulation of HY5 transcription (Jenkins, 2014). HY5 is shown to regulate numerous gene targets of the UV-B photomorphogenic response and *hy5* mutants are hypersensitive to elevated UV-B levels (Brown & Jenkins, 2008). HY5 also regulates other transcription factors involved in UV-B response such as MYB12, which is involved in flavonoid biosynthesis (Stracke *et al.*, 2010).

The fine balance of UVR8 mediated UV-B response is regulated by two closely related repressor proteins RUP1 and RUP2 (REPRESSOR OF UV-B PHOTOMORPHOGENESIS). RUP1 and RUP2 are WD40-repeat proteins whose WD40 sequence is highly similar to that of COP1. *RUP1* and *RUP2* are transcriptionally active under UV-B exposure in a UVR8, COP1 and HY5 dependent manner (Gruber *et al.*, 2010). RUP1 and RUP2 are thought to negatively regulate UVR8 by displacing COP1 at its binding site and causing redimerisation of UVR8 (Heijde & Ulm, 2013). Redimerisation of UVR8 is important, as degradation and *de novo* synthesis do not play a significant role in regeneration of UVR8, and the resulting dimer must be available to undergo further activation from UV-B (Heijde & Ulm, 2013). A double mutant *rup1 rup2* was found to be hyperresponsive to UV-B resulting in increased HY5 and CHS gene expression and elevated flavonoid levels, granting better acclimation and protection to UV-B stress (Gruber et al., 2010). Conversely over expression of RUP2 resulted in suppression of

HY5 and CHS in response to UV-B and decreased stress response and acclimation (Gruber et al., 2010).

The major regulator of downstream signalling from UVR8 is HY5 (Stracke et al., 2010). It has also been found to interact with a protein HYH (HY5 HOMOLOG) which has similar amino acid sequence identity (Holm *et al.*, 2002). Rapidly induced upon UV-B exposure and in a UVR8-COP1 dependent manner they are proposed to regulate many of the UV-B transcriptional responses (Holm et al., 2002, Stracke et al., 2010, Favory et al., 2009). HY5 itself is required for the expression of the repressors RUP1 and RUP2 and establishment of a negative feedback loop, but also activates COP1 expression (Favory et al., 2009, Gruber et al., 2010). It binds to its own promoter as well as those for COP1, RUP1, RUP2, CHS and MYB12 (Binkert *et al.*, 2014). Particularly important is the regulation of CHS in response to UV-B by HY5 as this represents the first committed step in the production of flavonoid compounds (Dao *et al.*, 2011). HY5 accomplishes this by binding an ACGT containing element (ACE) in the promoter of CHS (Stracke et al., 2010). In conjunction with this HY5 may also bind ACE elements in the MYB12 promoter which regulates CHS expression and a range of other genes involved in the synthesis of flavonoid compounds (Stracke *et al.*, 2007).

Although a basic understanding of the UV-B response involving UVR8 has been established, much is still yet to be understood. In particular further work on how UVR8 and its associations induce the UV-B morphological response and the signal transduction that occurs to activate downstream mechanisms involved in UV tolerance and acclimation.

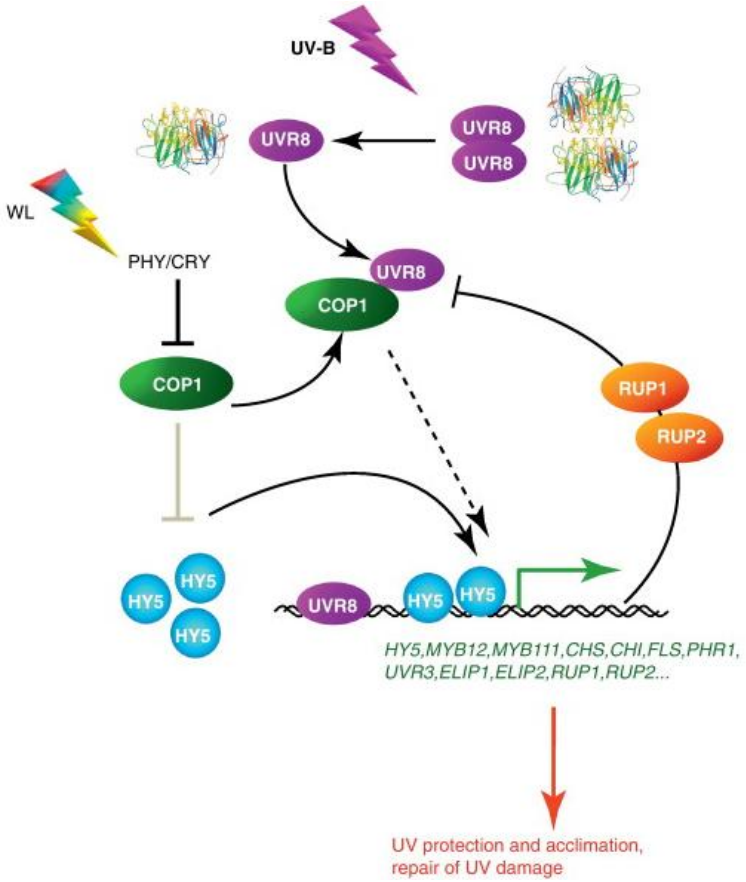



Figure 1.3: Model of UVR8-mediated signalling. Under light (WL) conditions devoid of UV-B, UVR8 is present mainly as a homodimer. COP1 represses photomorphogenesis by promoting degradation of HY5 (and other promotive transcription factors), but is under the negative control of light-activated phytochromes and cryptochromes. In the presence of UV-B radiation, UVR8 monomerises and interacts with COP1. The bZIP transcription factor HY5 is stabilised and UV-B-responsive genes are activated. These include genes encoding proteins of importance for UV protection (e.g. phenylpropanoid biosynthesis pathway, including CHS and FLS) and damage repair (e.g. photolyases PHR1 and UVR3), but also the RUP1 and RUP2 proteins, which constitute negative feedback on UVR8 activity involving direct protein–protein interaction. Abbreviations: *CHI*, *CHALCONE ISOMERASE*; *CHS*, *CHALCONE SYNTHASE*; CRY, cryptochrome; COP1, CONSTITUTIVELY PHOTOMORPHOGENIC 1; *ELIP1* and *ELIP2*, *EARLY LIGHT-INDUCIBLE PROTEIN 1* and *2*; *FLS*, *FLAVONOL SYNTHASE*; HY5, ELONGATED HYPOCOTYL 5; MYB12 and MYB111, MYB DOMAIN PROTEIN 12 and 111; *PHR1*, *PHOTOLYASE 1*; PHY, phytochrome; RUP1 and RUP2, REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1 and 2; UV-B, ultraviolet-B radiation; UVR3, UV REPAIR DEFECTIVE 3; UVR8, UV RESISTANCE LOCUS 8; WL, white light (From: Heijde & Ulm, 2012).

### 1.4.2 Non-Specific UV-B responses

The absorption of UV-B by a large range of compounds means that a variety of non-specific UV-B responses can occur upon irradiance. This is particularly the case during high fluence UV-B which induces signalling transduction pathways also associated with responses to pathogens, wounding and herbivory (Jordan, 2010).

This non-specific response is thought to be mediated mainly through the induction of ROS upon high UV-B irradiance, while signalling was shown to be inhibited by antioxidants and superoxide scavengers (Mackerness et al., 2001, Surplus *et al.*, 1998). ROS are a major signal in the UV-B response yet the exact origin of ROS production to UV-B is unknown and may involve a variety of mechanisms from damage to photosynthetic and respiratory machinery, lipid membranes damage or NADPH superoxidase (Jordan, 2010, Jenkins, 2009). Plants respond to increases in ROS from UV-B by enhancing antioxidant systems and ROS scavenging enzymes such as ascorbate isomerise and superoxide dismutase (Jenkins, 2009). While ROS induces a large number of genes it has little effect on the flavonoid pathway gene CHS that mediates the flavonoid acclimation responses (Gadjev *et al.*, 2006, Jenkins, 2009).

The increased activation of ROS via UV-B may stimulate the defensive pathways including the induction of jasmonate, salicylate, and ethylene (Jordan, 2010). These intermediates are then able to induce further gene expression changes (Jordan, 2002). Importantly the activation of stress and defence related intermediates by UV-B could lead to a variety of responses through Mitogen activated protein kinase (MAPK) signalling.

DNA damage may also impact on the induction of the non-specific UV-B response. DNA is particularly susceptible to UV-B as it absorbs strongly within the UV-B wavelength. Cyclobutane pyrimidine dimer formation has been associated with a UV-B responses (Giordano *et al.*, 2004, Kucera *et al.*, 2003). Specific UV-B responses, including the induction of β-1, 3-glucanase in French bean, were shown to be activated in response to DNA damage (Kucera et al., 2003). When the damage is repaired via photo-repair mechanisms the induction is blocked indicating that DNA damage is a primary signal for β-1, 3-glucanase transcription. Flavonoid synthesis was not seen to be affected by DNA damage however (Kucera et al., 2003).

While UVR8 mediated signalling is required for the perception and acclimation to UV-B light, a response has been identified, independent of UVR8, induced by acute UV-B stress (González Besteiro *et al.*, 2011). This second response occurs via the MAPK's, specifically the MKP1-regulated MAPK pathway. In the model plant *Arabidopsis* it was shown that an *mkp1* mutant is hypersensitive to acute UV-B stress while *uvr8* was not. Also *uvr8* is impaired under UV-B acclimation while *mkp1* is not (González Besteiro et al., 2011). The results showed that there are two major pathways responsible for the effective UV response in plants, with UVR8 regulating UV-B induced photomorphogenisis while a separate MAPK pathway is involved in acute UV-B stress.

Regulation and signalling involved in the MKP1 mediated pathway in response to UV-B stress is yet to be fully understood, but two other MAP kinase proteins MKP3 and MKP6 are known to be involved. MKP3 and MKP6 are activated in response to UV-B and are thought to be negatively regulated via MKP1. UV-B hypersensitivity of the *mkp1* mutant is thought to be caused via misregulation of MKP3 and MKP6, a view further strengthened by *mkp3* and *mkp6* mutants that exhibit elevated UV-B tolerance (González Besteiro et al., 2011). Interestingly MPK1 is phosphorylated and stabilised in response to UV-B stress potentially via MKP3 and MKP6, demonstrating an intriguing mechanism of interplay and control (González Besteiro & Ulm, 2013, Park *et al.*, 2011).

## 1.5 Photoperception of Non-UV-B light

Plants rely on light for photosynthesis so their perception of the light environment is often crucial for efficient development. Plants use specific photoreceptors not only for UV-B sensing but also in order to sense many factors including the intensity, spectral composition and direction of light in their environment. The Phototropins and Cryptochromes are able to sense light within blue wavelengths while the Phytochromes sense light within the red / far-red wavelengths (Fig. 1.4). A specific UV-B receptor, UVR8 senses UV-B wavelengths as discussed earlier (Fig. 1.3). Plants are largely anchored to the substrate they grow on so the

perception of the light environment and the ability to mediate developmental changes in response is particularly important for optimal growth and survival.

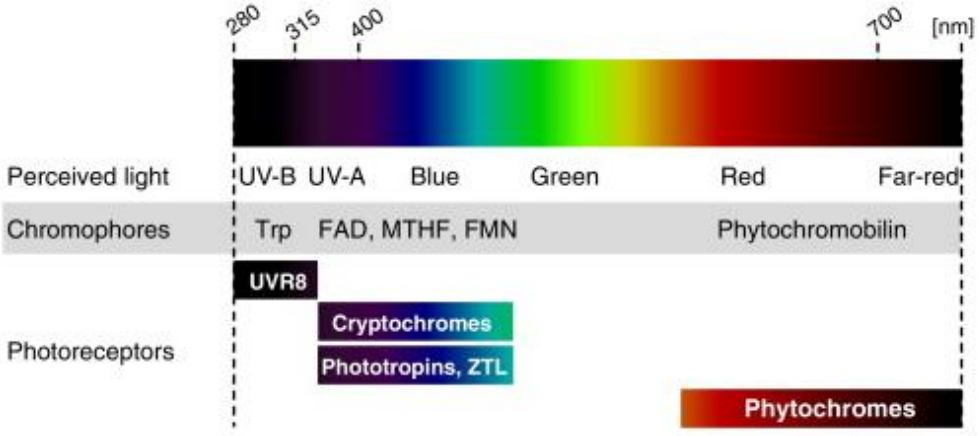



Figure 1.4: Photoreceptor-mediated light perception in higher plants. Plant photoreceptors perceive information from a large part of the light spectrum. UVR8 is the only UV-B photoreceptor identified to date. Tryptophan's (Trp) intrinsic to UVR8 were postulated to provide a 'UV-B antenna', with a major role identified for tryptophan-285. Various proteins harbour chromophores able to absorb light in the UV-A/blue part of the spectrum. Cryptochromes bind Flavin Adenine Dinucleotide (FAD) and methenyltetrahydrofolate (MTHF) as chromophores. Phototropins and the Zeitlupe (ZTL) proteins bind Flavin Mononucleotide (FMN) chromophore through their LOV (light, oxygen or voltage) domains. Phytochromes are red/far-red photoreceptors, also involved in some blue light responses, which use the plant-specific chromophore phytochromobilin, a linear tetrapyrrole (From Heijde and Ulm (2012).

### 1.5.1 Cryptochrome

Cryptochromes sense light within the UV-A / Blue light part of the spectrum and subsequently impact a diverse range of developmental processes in plants from de-etiolation to flower induction and circadian rhythm (Chen *et al.*, 2004, Chaves *et al.*, 2011, Li & Yang, 2007). Cryptochromes are structurally related to DNA-photolyases but do not possess the photolyase function. These photolyases are particularly important in repairing DNA damage due to UV-B radiation and are induced by UV-A / blue light (Sancar, 2003).

Cryptochromes were first identified in mutant *Arabidopsis* that were deficient in hypocotyl elongation under blue light (Ahmad & Cashmore, 1993, Koornneef *et al.*, 1980). The first cryptochrome was labelled CRY1 while a homolog was also identified based on sequence homology and labelled CRY2. These two cryptochromes have since been found to be widely distributed throughout many plant species (Chaves et al., 2011). CRY1 and 2 have both overlapping and distinct functions in response to light (Li & Yang, 2007, Chaves et al., 2011). CRY1 is stable in high light environments (Ahmad & Cashmore, 1993), while CRY2 is light liable and responds preferentially to low light (Yu *et al.*, 2007).

Cryptochrome photoperception and subsequent signalling is diverse and affects many biological functions, yet its signalling response partially overlaps with that of the UV-B response through the photomorphogenic pathway. Both CRY1 and CRY2 have been shown to interact with the E3 ubiquitin ligase COP1 and in the case of CRY2 this interaction may regulate flowering initiation by preventing degradation of constans (CO) protein (Liu *et al.*, 2008). Under high irradiance the induction of the phenylpropanoid pathway transcription factors such as HY5 were also induced in a cryptochrome dependent manner (Kleine *et al.*, 2007). COP1 and HY5 are both important regulators of the UV-B phenylpropanoid response.

### 1.5.2 Phototropins

Phototropins absorb light within the blue wavelength-band similar to the cryptochromes (Fig. 1.3). However, where cryptochromes regulate photomorphogenic responses the phototropins serve more to promote the photosynthetic efficiency of plants and promote growth (Christie, 2007). Phototropins are the regulators of phototropism whereby plants may grow towards (positive) or away (negative) from a light source. In *Arabidopsis* two phototropins are present, PHOT1 and PHOT2 which regulate phototropism in response to blue light (Sakai *et al.*, 2001). Under high blue light irradiance both PHOT 1 and PHOT 2 work to regulate phototropism while phot1 solely regulates phototropism under low blue light irradiance (Liscum & Briggs, 1995, Sakai et al., 2001). Stomatal opening is also controlled in a phot1/phot2 dependent manner and allows for regulation of $CO_2$ uptake and water

transpiration under blue light together with chloroplast movement allowing for efficient manipulation of photosynthesis (Kinoshita *et al.*, 2001, Sakai et al., 2001, Takemiya *et al.*, 2005).

In the basal plant *Physcomitrella patens* four phototropins were identified and regulate chloroplast movement under blue and red light (Kasahara *et al.*, 2004). The fern *Adiantum capillus-veneris* also has two phototropins that regulate chloroplast movement but also a novel photoreceptor neochrome (neo) (Suetsugu *et al.*, 2005). This photoreceptor is similar to the phototropins, with a phytochrome photosensory domain fused to an entire phototropin receptor, and regulates phototropism and chloroplast movement in *Adiantum* under red and blue light (Kawai et al., 2003).

### 1.5.3 Phytochrome

Phytochrome senses light within the red / far-red wavelengths which although not photosynthetically active plays important roles in plant shade avoidance, germination and seedling de-etiolation (Possart *et al.*, 2014, Chen et al., 2004). Phytochromes exist in the inactive $P_r$ and active $P_{fr}$ forms. $P_r$ absorbs light within red wavelengths ($\lambda_{max}$, 666 nm) while $P_{fr}$ absorbs within the far red wavelengths ($\lambda_{max}$, 730nm). The absorption of light by either converts them to the opposing form, creating a dynamic equilibrium between the two (Mancinelli, 1994). This characteristic absorption by photochromes allows for its unique functions in regulating shade avoidance and light competition from neighbouring plants (Casal, 2013).

Signal transduction from phytochrome perception of light begins with translocation from the cytosol to the nucleus (Possart et al., 2014). The two main phytochromes phyA and phyB rely on separate mechanisms for this to occur. PhyA binds FAR-RED ELONGATED HYPOCOTYL 1 (FHY1) and FHY1-LIKE (FHL) which contain a nuclear localisation signal and mediate phyA transfer to the nucleus (Hiltbrunner *et al.*, 2006). phyB does not enter the nucleus through the same mechanism and is thought to have a nuclear localisation signal that is unmasked

upon activation (Chen *et al.*, 2005). This occurs alternatively or together with transcription factor mediated transport (Pfeiffer *et al.*, 2012).

Phytochrome interacts with the same regulators that are involved in the UV-B response and cryptochrome response such as HY5, COP1 and SPA1 (Chen & Chory, 2011). In particular and in line with the general response to white light, phytochromes are involved in this response through the reorganisation of the COP1/SPA1 complex (Sheerin *et al.*, 2015). Light activated phyA and phyB are able to bind SPA1 which disrupts its association to COP1 and thus stabilises photomorphogenic targets of the COP1/SPA1 complex such as HY5 (Sheerin et al., 2015).

## 1.6 Plant pigments

Plants produce an array of diverse compounds including plant pigments which are able to absorb at different wavelengths of light, often resulting in the different colourations we observe (Davies, 2009). The most noticeable example of plant pigments is the colouration of flowers and their use as pollinator attractors, yet they serve a much more diverse range of functions from driving photosynthesis to protection from abiotic stress. The exact function of each plant pigment depends on its structure and the most important of the plant pigments can be limited to chlorophylls, carotenoids, betalains, and flavonoids.

### 1.6.1 Chlorophylls

Chlorophylls are responsible for the green colour observed in vegetative tissues and are required for the harvesting and transduction of light energy for photosynthesis. Their importance in this role cannot be overstated.

Chlorophylls belong to the tetrapyrroles, which contain related compounds such as haem and vitamin B12. Chlorophylls are distinguished by a magnesium ion within the tetrapyrrole ring and a fifth so-called isocyclic ring (Davies, 2009, Jansson, 1994). Plant photosynthesis relies on two major chlorophyll structures, chlorophyll *a* and chlorophyll *b* (Fig 1.5). The structure of chlorophyll allows it to strongly absorb light within the blue and red light

wavelengths resulting in the distinct green colour observed. Chlorophyll also emits red under blue light which can give key insight into the photosynthetic machinery (Schreiber, 2004).

The distribution of chlorophyll *a* and *b* differ in that chlorophyll *a* is associated with the photosystems I and II while chlorophyll *b* is associated with the light harvesting complexes (LHCs). The ratio of *a*/*b* varies in plants and particularly between shade adapted and light exposed plants. Shade adapted plants tend to have higher amounts of LHCs than light exposed plants so the ratio favours chlorophyll *b.* In general shade adapted plants contain an *a*/*b* ratio of 2.0-2.8 while full sun exposed plants have a ration of 3.5-4.9 (Davies, 2009, Boardman, 1977)

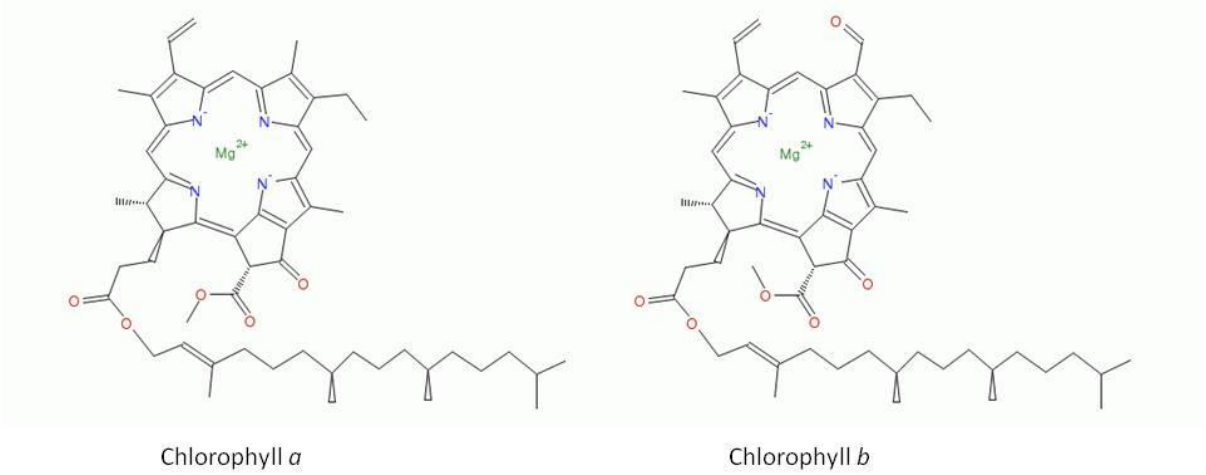



Figure 1.5: Structure of chlorophyll *a* and *b*

The biosynthesis of chlorophyll must be tightly regulated as accumulation of its intermediates can cause toxic effects within the cell. Chlorophyll, like the other tetrapyroles, is synthesised from eight molecules of aminolevulinic acid (ALA). ALA is synthesised from glutamine via the $C_5$ pathway. After ALA synthesis, a number of enzymes and intermediates are involved before the final chlorophyll structure is derived; summarized in Beale (1999), and Davies (2009).

Chlorophylls absorb light for photosynthesis but if the absorbed energy is not used it must be dissipated, otherwise it can result in the formation of damaging ROS. The most common production of ROS from chlorophyll is through energy transfer to oxygen to form singlet oxygen (Asada, 2006). The singlet oxygen generated can cause a cascade of damaging reactions catalysed by free radicals resulting in disruption to proteins and nucleic acids ultimately cumulating in plant cell death if left unchecked. In order to limit singlet oxygen and free radical production plants employ the use of antioxidants, superoxide dismutase, and accessory pigments such as carotenoids to dissipate excess energy from chlorophylls (Asada, 2006, Davies, 2009).

### 1.6.2 Carotenoids

Carotenoids form a large subgroup of isoprenoid compounds found within animals, microbes and plants. A diverse group of over 700 compounds the carotenoids provide distinct red, orange and yellow colours. Carotenoids are well known for their essential roles in plant photosynthesis, as well as a nutritional requirement for vitamin A production in humans.

Most carotenoids comprise a tetraterpene ($C_{40}$) backbone but carotenoids with $C_{30}$ and $C_{50}$ are also produced by certain bacteria (Walter & Strack, 2011). The colour properties of carotenoids are due to double bond introduction into this carbon backbone. The backbone is linear, with modification of terminal ends responsible for the diversity of compounds. The most common modification is the formation of a β-ionone terminal ring structure such as those in β-carotene and the xanthophylls (Fig. 1.6). Those carotenoids with terminal β-ionone ring structures also form the precursors for vitamin A production making them essential dietary components of those organisms that cannot synthesise vitamin A.

In general carotenoid biosynthesis begins with the first committed step of phytoene production through the condensation of geranylgeranyl diphosphate (GGPP). Phytoene then undergoes multiple desaturation reactions to produce tetra-*cis*-lycopene, followed by isomerisation to produce all-*trans*-lycopene. Lycopene can then be cyclised to produce α or

β-carotene followed by further modification to produce xanthophylls (Davies, 2009, Cunningham Jr & Gantt, 1998).

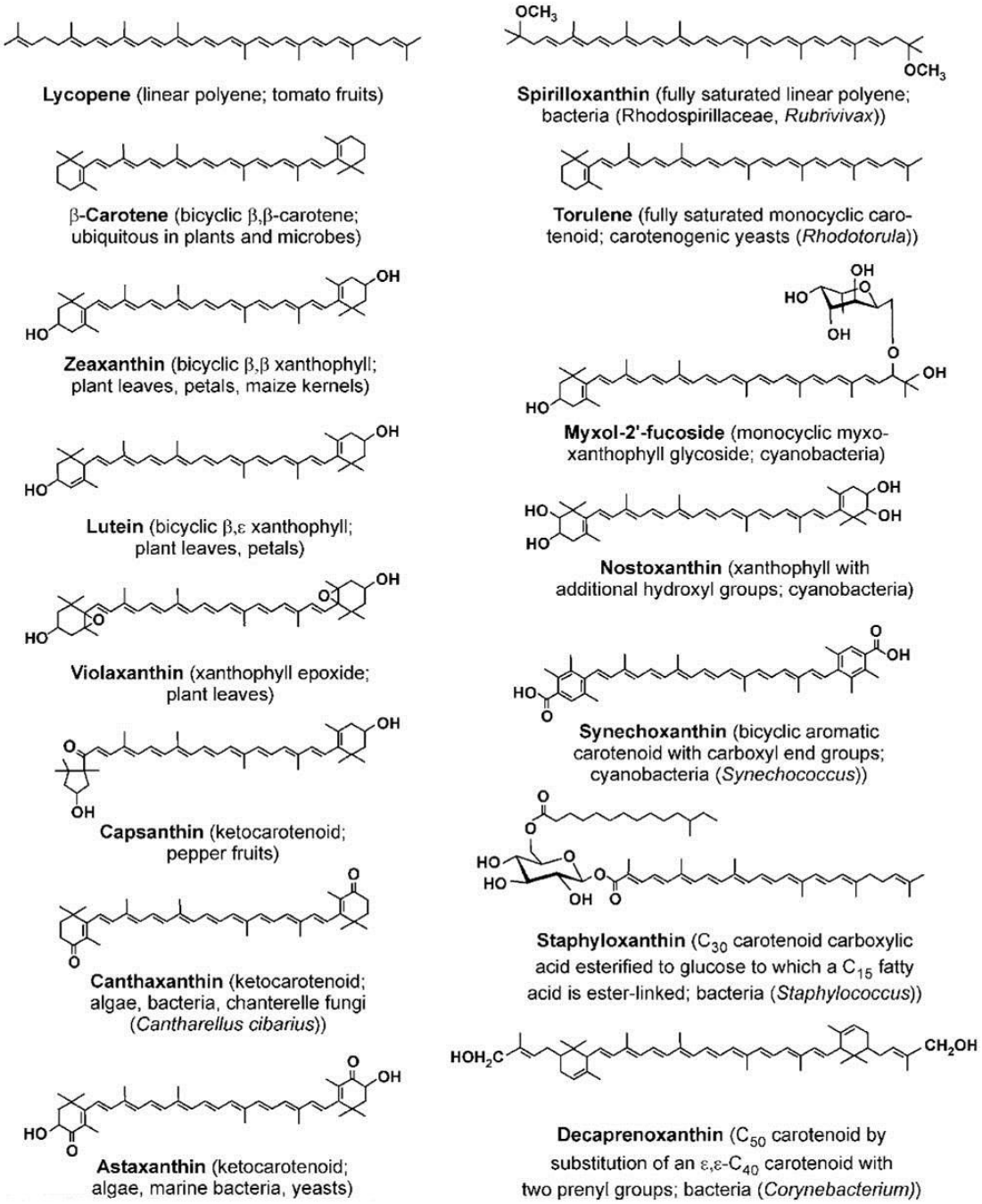



Figure 1.6: Structural diversity of selected carotenoids from various organisms. A structural classification is given, followed by major occurrences of the compound (From Walter and Strack (2011).

Carotenoids are found in all photosynthetic organisms where they play important roles mainly through light harvesting and photoprotection. Contribution to light harvesting occurs

through their absorption of light energy in a spectrum where chlorophyll absorption is less efficient. Carotenoids form protein complexes with PSII allowing for correct folding and stability. Carotenoids also form protein-pigment complexes with LHC antenna and energy transfer to chlorophyll (Cazzonelli, 2011, Davies, 2009).

Carotenoids are not only involved in the efficient transfer of light energy for photosynthesis but also in the photoprotection of this machinery. Under high light the photosynthetic machinery must be able to efficiently dissipate excess energy and prevent the formation of damaging reactive oxygen and free radicals. Carotenoids regulate this through photoprotective mechanisms including quenching triplet chlorophylls. They also play important roles in dissipation of excess energy via non-photochemical quenching (NPQ), mediated by xanthophylls, and ROS scavenging by carotenoid antioxidants (Cazzonelli, 2011, Davies, 2009).

### 1.6.3 Betalains

Betalains are nitrogen containing pigments present in most plants of the order Caryophyllales (Gandía-Herrero *et al.*, 2014). Betalains exist as the yellow betaxanthins and violet betacyinins with maximum absorptions of 480 nm and 536 nm respectively. Betalamic acid is the light sensing chromophore. The presence of betalains in plants is mutually exclusive to that of anthocyanin (Brockington *et al.*, 2011). Although not known for any UV protectant functions, the betalains have gained attention due to their antioxidant, antiradical and potential cancer preventative activities (Gandía-Herrero et al., 2014). The origin of betalains and the lack of anthocyanins in the plants that produce them are still unclear. However, it may be that key genes in the flavonoid pathway are inactive for anthocyanin synthesis or a lack of precursor for betalain synthesis limits production in non-producing species (Sakuta, 2014).

### 1.6.4 Proanthocyanidins

Proanthocyanidins or condensed tannins are colourless pigments comprised of oligomers of flavon-3-ols. They are known for their use as tanning agents of animal skins and flavour

compounds in teas and wines while also having important plant functions for microbial, insect and herbivore protection (Dixon *et al.*, 2005). Proanthocyanidins comprises of polymers of 2-10 flavon-3-ol units whose final structure depends largely on the starting units stereochemistry and hydroxylation (Dixon et al., 2005, Davies, 2009). Oligomers are comprised largely of C4-C8 linkages between 2,3-*tran*-flavonoids or 2,3-*cis*-flavonoids (Dixon et al., 2005). The biosynthesis of the flavon-3-ol starting structures are well known through studies of the flavonoid biosynthetic pathway, while the condensation reactions forming the proanthocyanidin oligomers less so (Xie & Dixon, 2005, Dixon et al., 2005).

Proanthocyanidins are implicated in ROS scavenging and antioxidant activities (Santos-Buelga & Scalbert, 2000). Proanthocyanidins were shown to scavenge $O_2^{\cdot -}$, $OH^{\cdot}$ in aqueous solutions as efficiently as the flavonol quercetin in some cases (Plumb *et al.*, 1998). The influence of polymerisation on antioxidant activity is unclear with contrasting results found for increases in polymerisation although galloylation was found to increase the scavenging activities of proanthocyanidins (Plumb et al., 1998, Santos-Buelga & Scalbert, 2000). The exact role that the proanthocyanidins play in plant antioxidant and free radical scavenging is still unclear but they may play a role under specific conditions.

## 1.7 Flavonoids

Flavonoids are a group of secondary metabolites with a broad range of biological functions. They serve as signalling molecules, abiotic and biotic stress protectants, facilitators of auxin transport, and as ROS scavenging compounds (Buer *et al.*, 2010, Agati *et al.*, 2012, Andersen & Markham, 2010, Davies, 2009). However the most known function is that of flower colouration providing visual cues and attraction to pollinators, while also being a desirable visual trait in floriculture (Davies *et al.*, 1997). Although known to have diverse functions, the role of flavonoids in UV-B protection, ROS scavenging and antioxidant activity are of particular interest. Here we will focus on the main flavonoid groups; chalcones, flavones and flavonols, anthocyanins and deoxyanthocyanidins.

### 1.7.1 Chalcones

Chalcones are the first core compound in the flavonoid pathway and are synthesised after condensation of three malonyl-CoA molecules onto *p*-coumaroyl-CoA, catalysed by chalcone synthase (CHS) (Ferrer *et al.*, 2008). A related enzyme, stilbene synthase (STS), catalyses a similar reaction to form stilbene compounds. The similarity of structure and function between the two enzymes indicated that the two had differentially evolved from a non-iterative β-ketoacyl ACP synthase (KASIII) involved in fatty acid biosynthesis (Austin & Noel, 2003). The chalcone product is utilised further in the production of flavanones, catalysed by chalcone isomerise (CHI).

Chalcone compounds have been shown to exhibit a variety of functions and have gained specific interest for their antioxidant and anticancer properties (Sivakumar *et al.*, 2011, Bandgar *et al.*, 2010). Synthetic and natural chalcones also exhibit antifungal, antimicrobial and anti-inflammatory properties (Nowakowska, 2007). These effects were largely based on human diet and the effective activity of chalcones in plants may be limited, as they are used as the building blocks for the flavonoid compounds and may be quickly utilised through the flavonoid pathway. The up-regulation of the chalcone synthase enzyme is therefore particularly important in the activation of chalcone synthesis and subsequently flavonoid synthesis, in response to a variety of plant stresses including UV light (Dao et al., 2011).

### 1.7.2 Flavones and Flavonols

The flavones and flavonols are found widely distributed in the plant kingdom and can be responsible for white and yellow colour in flowers. Interestingly flavones are absent from almost all Brassicaceae species while the flavonols are present along with the other flavonoid compounds (Martens & Mithöfer, 2005). The flavones and flavonols represent an ancient class of compounds often associated with the evolution of plants onto land and fossil records for flavones date back 17-25 million years (Martens & Mithöfer, 2005, Pollastri & Tattini, 2011, Niklas & Ginnasi, 1978). Both flavones and flavonols have important plant functions, not least of which is their ability to absorb wavelengths within the UV-B spectrum (280-320 nm) and act as antioxidants. Structurally they are similar with the primary

difference of oxygenation at C-3 in the flavonols (Markham, 2012). Common flavones and flavonols including structural differences are shown in Figure 1.7.

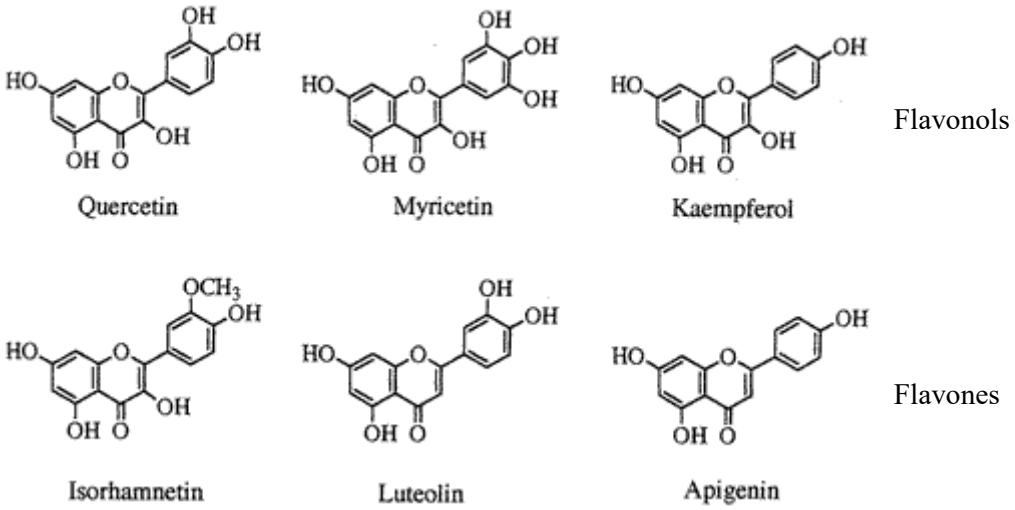



Figure 1.7: Structures of the main flavonols and flavones (From. Crozier *et al.* (2000)).

Flavone synthesis occurs at the branch point between anthocyanin and 3-Deoxyanthocyanidins (Fig. 1.8). Flavones are produced via a (2S)-flavanone substrate by the enzymes flavone synthase I (FNSI) or flavone synthase II (FNSII). Most plants contain FNSII, a membrane bound NADPH-dependent Cyt P450. The FNSI enzyme, a 2OG-dioxygenase, is much less common and only appears to occur within the Apiaceae (Martens & Mithöfer, 2005). FNSI also has high sequence similarity to flavanone 3-hydroxylase (F3H), which is thought to be due to the early duplication of F3H and functional diversification which led to the origin of FNSI (Gebhardt *et al.*, 2005). Flavonols are synthesised from (2R,3R)-dihydroflavonol substrate by a 2OG-dioxygenase enzyme, flavonol synthase (FLS), first identified in petunia (Holton *et al.*, 1993). The dihydroflavonol substrate is formed from flavanone, catalysed by F3H (Fig. 1.8).

### 1.7.2.1 Function of Flavones

Like many of the other flavonoids, flavones are able to absorb wavelengths within the range of UV-B and have been proposed to be involved in the screening of these damaging

wavelengths (Martens & Mithöfer, 2005). Indeed, *O*-methylated flavones were found to have protective UV screening functions within two *Gnaphalium* species (Cuadra & Harborne, 1996). The results indicated that increases of flavone compounds within the epidermal layer in response to UV-B irradiation may confer plant protection via a UV screening mechanism. In the liverwort *M. polymorpha*, exposure to enhanced UV-B was found to alter the ratio between luteolin to apigenin flavone glycosides. When exposed to UV-B the ratio between the two shifted to favour an increased production of luteolin, which was associated with an improved level of antioxidant defence rather than UV-B screening (Markham *et al.*, 1998a). Similar response in a UV-B tolerant rice cultivar was observed with increased antioxidant flavones (Fig. 1.9), in favour of flavones with UV screening properties (Markham *et al.*, 1998b). Plant species may vary in their use of flavone or flavonol compounds, especially glycoside derivatives which absorb strongly between 280-320 nm, for UV screening or antioxidant function whether through changes in ratios of compounds, or localisation to epicuticular and epidermal regions (Harborne & Williams, 2000).

Flavones are also involved in the interaction between organisms such as legumes and nitrogen fixing rhizobia. Flavonoids, including flavones, are exuded from the roots of the host plant and are specifically recognised by rhizobial bacteria for the initiation of nodulation factors (nod). Nod factors are in turn then recognised by the plant and the symbiotic signalling leading to nodulation and nitrogen fixation established (Redmond *et al.*, 1986, Zhang *et al.*, 2009). Flavones, along with many other flavonoids, have also been noted for their protection against a range of insect pests, mainly through their feeding deterrent activities (Simmonds, 2003).

### 1.7.2.2 Function of the Flavonols

Flavonols are proposed to perform a variety of functions within the plant from UV screening and antioxidant scavenging to modulating auxin transport and DNA protection (Pollastri & Tattini, 2011). Flavonols are implicated in the evolution of early land plants, aiding their adaption to land through UV screening of damaging light. Analysis of their functional role in plants has led to the hypothesis that flavonols may not have been as important in UV screening functions, but favoured more in the reduction of reactive oxygen species generated by exposure to harsh light conditions (Pollastri & Tattini, 2011). Flavones likely

arose prior to flavonols to function in UV-B screening, so flavonols may have taken additional roles such as ROS scavenging.

The flavonol synthesis genes are present in extant relatives of the first land plants, the bryophytes, yet the proposed green algae ancestors lack flavonoid production (Rausher, 2006). Flavonols may have been favoured over the mycosporin like amino acids utilised by green algae, as they can perform multifunctional roles including UV screening and antioxidant reduction. Flavonols are able to perform UV screening roles due to their absorption of wavelengths between 290-320nm. The significance of this function is disputed however, with induction of flavonols that exhibit higher antioxidant function (Fig. 1.9) over UV-B screening ability in response to UV-B and light stress (Agati *et al.*, 2011, Gerhardt *et al.*, 2008). The function of flavonols as antioxidants is further supported by their accumulation in mesophyll cells in response to high light stress (Agati *et al.*, 2009). It may be, however, that epidermal cells must not only be able to screen damaging UV wavelengths from the underlying cells, but also cope with oxidative damage from such light and thus protective flavonols within this layer need both UV screening and antioxidant functions to be effective (Stafford, 1991, Pollastri & Tattini, 2011).

A DNA protective function of flavonols is also proposed as transgenic Arabidopsis overexpressing flavonol synthase show less DNA damage than wild type under UV-B conditions (Emiliani *et al.*, 2013). Protection was associated with increased flavonol levels in the nucleus that may directly protect against DNA damage (Emiliani et al., 2013).

### 1.7.3 Anthocyanins

Anthocyanins are widespread and abundant in plants and well known for their absorption of light in the longer wavelengths resulting in a mainly red spectrum of colouration in plant tissue. Apart from pigmentation, the function of anthocyanins in plants is diverse and includes responses to stress and attenuation of high light and UV-B by antioxidants (Hatier & Gould, 2009). In brief, anthocyanins are produced in the flavonoid pathway first from dihydroflavonol conversion to leucoanthocyanins via an NADPH dependent reduction by dihydroflavonol 4-reductase (DFR). Leucoanthocyanins are then converted by anthocyanidin

synthase (ANS) to form a 3-flaven-2,3-diol pseudobase which must be transported to the vacuole to be stabilised by O-glycosylation and undergo further secondary modification to form coloured anthocyanin products (Davies, 2009, Springob *et al.*, 2003).

Anthocyanins are well known for their role in photoprotection by absorbing excess light energy. As anthocyanins are predominantly contained within the vacuole the absorbance of excess light is contained and partitioned from the photosynthetic machinery, especially that of the chlorophylls (Gould, 2004, Hatier & Gould, 2009). The photoprotective ability of anthocyanins varies with different levels of protection in different species dependent of localisation, amount and the stage of tissue development (Hatier & Gould, 2009).

Anthocyanins are up-regulated in response to UV-B (Kusano *et al.*, 2011, Brandt *et al.*, 1995) and Arabidopsis mutants deficient in anthocyanin production show hypersensitivity to UV-B (Li *et al.*, 1993a). The protection from UV-B, as with other flavonoid compounds, is proposed to be due to antioxidant functions that anthocyanins possess. Any UV-B screening activity of anthocyanins would not account for a high level of protection as they are more commonly found in the vacuole of underlying cells rather than epidermal cells or cuticle layers (Hatier & Gould, 2009). The screening capacity of anthocyanins for other wavelengths may even be detrimental for UV-B protection as it filters out light required for the light dependent DNA repair enzyme photolyase, thus reducing repair of DNA damage caused by UV-B (Hada *et al.*, 2003).

The protection offered by anthocyanins in UV-B tolerance is most likely through their ability to act as free radical scavengers. The production of free radicals occurs largely due to excess energy from light including UV-B. Anthocyanins may absorb excess visible light of longer wavelength to first prevent the formation of such free radicals, and also scavenge any free radicals that are produced including those occurring from incident UV-B (Hatier & Gould, 2009). While anthocyanins are present in most higher plants today they are uncommon in the more ancient bryophytes (Lee, 2002) and may not have been involved in the defence against high light and UV-B by the first land plants.

### 1.7.4 3-Deoxyanthocyanins

3-Deoxyanthocyanins are relatively rare compound found in few species, but are common in moss and fern species (Iwashina, 2000). They lack the 3-hydroxylation found in the more common anthocyanins and subsequently absorb different wavelengths resulting in yellow, orange and bright red pigments (Davies, 2009). The study of 3-deoxyanthocyanidins has been mainly focussed on sorghum as it is a high producer of these compounds (Kayodé *et al.*, 2011). These compounds were found to have high antioxidant activities similar to those of the more common anthocyanins and may function in much the same way (Kayodé et al., 2011, Devi *et al.*, 2011). The *in planta* function and role in defence against UV is much less understood but given their antioxidant activity they may function to inhibit the formation of damaging levels of ROS produced in response to UV-B irradiance.

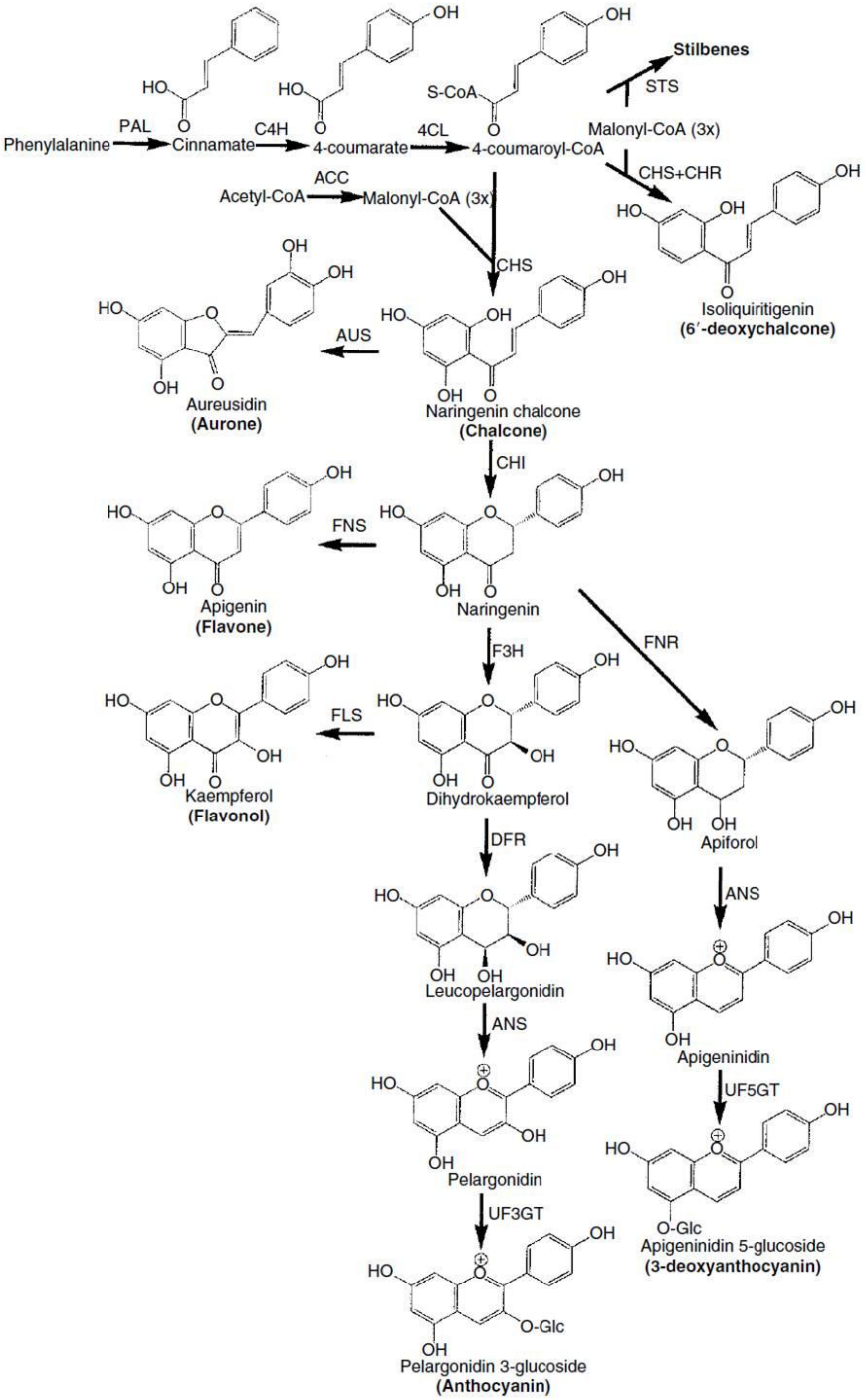



Figure 1.8: Schematic of a section of the flavonoid biosynthetic pathway. Enzyme abbreviations as follows: phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL), acetyl-CoA carboxylase (ACC), stilbene synthase (STS), chalcone reductase (CHR), chalcone synthase (CHS), aureusidin synthase (AUS), chalcone isomerise (CHI), flavone synthase (FNS), flavanone 3β-hydroxylase (F3H), flavonol synthase (FLS), flavanone 4-reductase (FNR), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), anthocyanin 3-O-glucosyltransferases (UF3GT), anthocyanin 5-O-glucosyltransferases (UF5GT). Only the routes to the production of flavonoids with 4'-hydroxylation are shown. The product of the ANS is shown in the traditional cation form (rather than the pseudobase) (From. Davies (2009)).

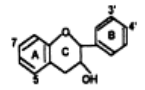
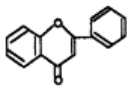
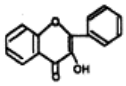
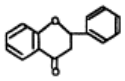
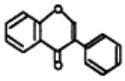
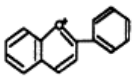
| Class | General structure | Flavonoid | Substitution Pattern | Dietary Sources | TEAC (mM) |
|---|---|---|---|---|---|
| Flavanol |  | (+)-catechin | 3,5,7,3′,4′-OH | Tea (camellia sinensis)[6] | 2.4 |
| | | (-)-epicatechin | 3,5,7,3′,4′-OH | Tea[6] | 2.5 |
| | | Epigallocatechin gallate | 3,5,7,3′,4′,5′-OH,3-gallate | Tea[6] | 4.75 |
| Flavone |  | chrysin | 5,7-OH | Fruit skins | 1.43 |
| | | apigenin | 5,7,4′-OH | Parsley, celery | 1.45 |
| | | rutin | 5,7,3′,4′-OH, 3-rutinose | Red wine[5] , buckwheat[7] citrus, tomato skin[8] | 2.4 |
| | | luteolin | 5,7,3′,4′-OH | Red pepper[11] | 2.1 |
| | | luteolin glucosides | 5,7,3′-OH, 4′-glucose | | 1.74 |
| | | | 5,4′-OH, 4′,7-glucose | | 0.79 |
| Flavonol |  | kaempferol | 3,5,7,4′-OH | Leek, broccoli, endives grapefruit, black tea | 1.34 |
| | | quercetin | 3,5,7,3′,4′-OH | Onion, lettuce, broccoli tomato, tea, red wine berries, olive oil, appleskin | 4.7 |
| | | myricetin | 3,5,7,3′,4′,5′-OH | Cranberry grapes, red wine | 3.1 |
| | | tamarixetin | 3,5,7,3′-OH,4′-OMe | | |
| Flavanone (dihydroflavon |  | naringin | 5,4′-OH,7-rhamnoglucose | Citrus, grapefruit | 0.24 |
| | | naringenin | 5,7,4′-OH | Citrus fruits | 1.53 |
| | | taxifolin | 3,5,7,3′,4′-OH | Citrus fruits | 1.9 |
| | | eriodictyol | 5,7,3′,4′-OH | Lemons[64] | 1.8 |
| | | hesperidin | 3,5,3′-OH,4′-OMe, 7-rutinose | Oranges[9] | 1.08 |
| Isoflavone |  | genistin | 5,4′-OH, 7-glucose | Soybean[10] | 1.24 |
| | | genistein | 5,7,4′-OH | Soybean[10] | 2.9 |
| | | daidzin | 4′-OH, 7-glucose | Soybean[10] | 1.15 |
| | | daidzein | 7,4′-OH | Soybean[10] | 1.25 |
| Anthocyanidin |  | apigenidin | 5,7,4′-OH | Colored fruits | 2.35 |
| | | cyanidin | 3,5,7,4′-OH,3,5-OMe | Cherry, raspberry, strawberry | 4.42 |

Figure 1.9: Classification, structure, food sources, and Trolox equivalent antioxidant activities (TEAC) of dietary flavonoids. Higher TEAC values reflect greater antioxidant capability. A free 3-hydroxyl group and 3′,4′-catechol (dihydroxy) structure, a 2–3 double bond, and a 4-oxo group endow the flavonoid with activity superior to isoforms lacking these features. Glycosidic substitution decreases TEAC (From Heim *et al.* (2002)).

## 1.8 Pigments of Green Algae

While plant pigments may play a role for UV-B protection in the first land plants they may also have been present in the aquatic ancestors of these plants. The proposed ancestors of the first land plants are those of the green algae which contain the pigments required for photosynthesis and light harvesting such as chlorophylls and carotenoids but whether they were able to also synthesise protective pigments is of particular interest. While the first land plants contain flavonoids for UV-B protection, green algae do not possess flavonoid compounds but rather use mycosporin-like amino acids (MAAs) (Gröniger & Häder, 2002, Rozema *et al.*, 2002, Xiong *et al.*, 1999).

MAAs are low molecular weight water soluble compounds with the ability to absorb wavelengths between 300 and 365 nm. Structurally the mycosporins consist of an aminocyclohexenone or an aminocycloheximine ring, carrying nitrogen or imino alcohol substituent's while MAAs carry amino acid substituent's (Fig 1.10) (Oren & Gunde-Cimerman, 2007, Shick & Dunlap, 2002). The biosynthesis in algae is thought to occur via the shikimate pathway although the exact mechanism is unknown (Shick & Dunlap, 2002).

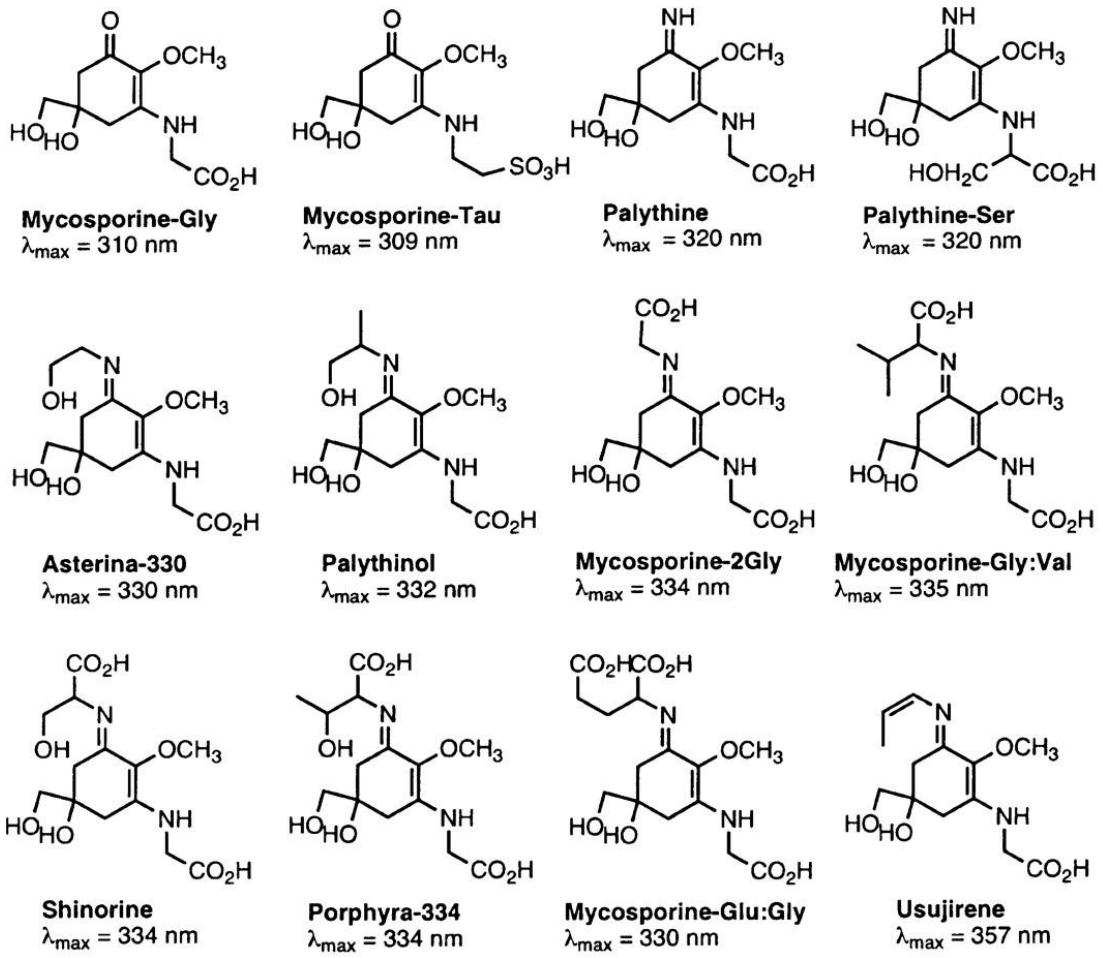



Figure 1.10: Molecular structures and wavelengths of maximum absorption ($\lambda_{max}$) of mycosporine-like amino acids. (From Shick and Dunlap (2002))

In algae MAAs function predominantly as UV protective and antioxidant compounds (Karsten *et al.*, 1999, Korbee *et al.*, 2005, Shick & Dunlap, 2002). MAAs perform this function through their ability to absorb UV wavelength within the UV-B spectrum (Fig. 1.10). As effective sunscreens they must not only be able to absorb UV energy but also dissipate this absorbed energy without generating ROS (Shick & Dunlap, 2002) were able to show that shinorine absorption under UV radiation did not result in free radical formation or fluorescence consistent with an efficient thermal dissipation of absorbed energy. The MAAs of *Phaeocystis antarctica* (predominately shinorine) are also shown to absorb UV energy without subsequent transfer to chlorophyll (Moisan & Mitchell, 2001). Interestingly plants do not contain the ability to synthesis any MAAs suggesting that the algal ancestor of the

first land plants may not have contained MAAs or that this was lost in favour of flavonoid compounds during the first colonisation of land (Shick & Dunlap, 2002).

## 1.9 Liverworts

Species resembling liverworts are proposed as the first plants to have inhabited the earth. *Marchantia polymorpha* is a simple thalloid liverwort and is ideal for the study of the evolutionary steps that led to the first colonisation of land (Ishizaki *et al.*, 2008). Its rapid growth, small size, ease of genetic transformation and clonal production makes them an excellent model organism. As the species retaining most similarity to the first land plants they have many unique features that distinguish them from the common higher plants of today.

### 1.9.1 Physiology of Liverworts

The dominant growth of the gametophyte of *M. polymorpha* is that of the thallus tissue. This tissue is generally several cell layers thick and in the case of *M. polymorpha* contains air chambers in the cells of the upper surface. The thallus is able to absorb water and nutrients from the atmosphere and much of its nutritional requirements are satisfied in this way allowing liverworts to grow in nutrient poor areas (Raven *et al.*, 2005). The liverworts have no functional stomata but instead utilise complex multicellular pores (Raven, 2002). These pores allow a greater area available for the diffusion of gas and $CO_2$ than would occur on thallus lacking such pores. This has been suggested to have given a selective advantage in $CO_2$ fixation and could result in at least a 20-fold increase in the gas-water interface for $CO_2$ exchange (Raven, 2002). Liverworts also lack proper vasculature systems for active transport throughout the plant and rely on relatively inefficient transport through the intercellular spaces and along the thallus surface (Shaw & Goffinet, 2000).

The thallus tissue grows in a largely branched structure from the apical notch, which can be easily identified at the end of the growing thallus. The thallus is comprised of a number of rhomboidal or polygonal areas known as areolae (Awasthi, 2005). The areola mark the edges

of the underlying air chamber and each has an air pore to allow for gas exchange. The surface of the areola is comprised of scales. The thallus itself is largely comprised of the epidermal, photosynthetic and storage regions (Fig. 1.11). The epidermal region consists of a thin layer of square shaped cells that contain very few chloroplasts. The cell walls are thickened and water proof and provide protective function for the underlying tissue. Below the epidermal layer is the photosynthetic layer which is largely contained within the air pores. The large air pores contain photosynthetic filaments as well as the surrounding tissue that is enriched in chloroplasts. The air pores in the chamber allow for respiration and gas exchange for the photosynthetic tissue. Below the photosynthetic region sits the storage region which contains cells that lack chloroplasts but are enriched in starch granules (Fig. 1.11). Cells in this region may also carry oil bodies or be filled with mucilage (Awasthi, 2005). Below this layer lies the specialised rhizoid structures.

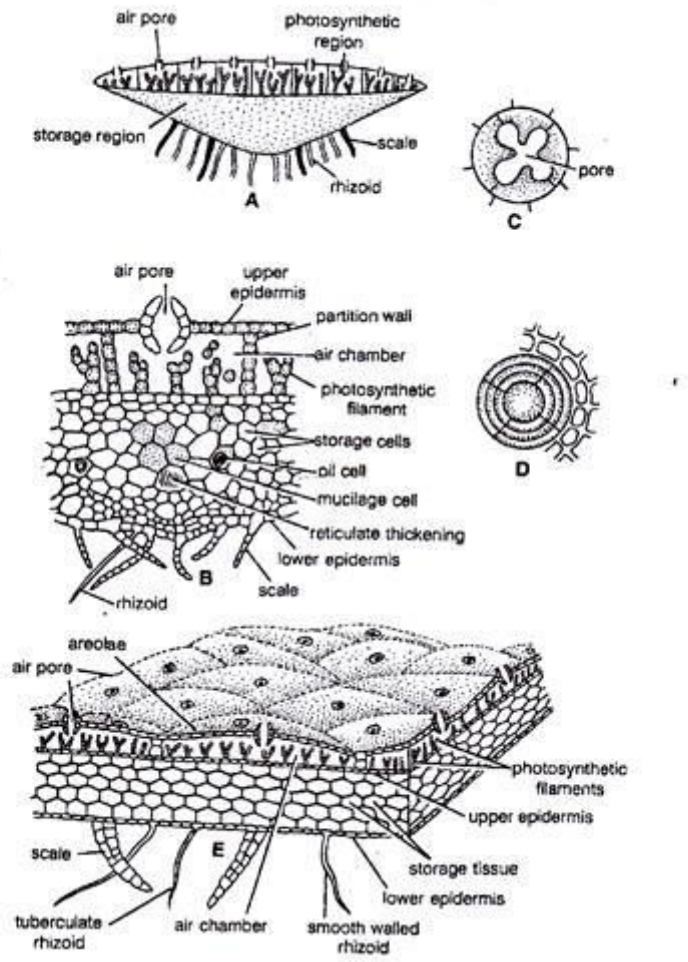



Figure 1.11: Marchantia internal structure of the thallus. A) Vertical transverse section of thallus. B) Transverse section of thallus (part cellular). C) Air pore as seen in the ventral view. D) Air pore as seen in the dorsal view. E) Transverse section of thallus in three dimensional view.

Many bryophytes, including liverworts, utilise specialised structures called rhizoids in order to anchor to a substrate (Jones & Dolan, 2012). While rhizoids are known to be important for the anchorage of liverworts, their involvement in water transport is much less well understood. Marchantiales utilise tuberculate rhizoids and smooth rhizoids (Cao *et al.*, 2014). The tuberculate rhizoids extend along the underside of thallus tissue parallel to the horizontal axis and converge upon the midrib region. Smooth rhizoids appear in clusters on mature thallus tissue and extend perpendicular to the thallus making contact with the substrate (Fig. 1.12) (Cao et al., 2014).

Tuberculate rhizoids display thick walls with a small diameter. They function largely to enhance the mechanical strength of the thallus but are also involved in desiccation tolerance and in some cases able to function as a water conducting system (Cao et al., 2014, Duckett *et al.*, 2014). In contrast the smooth rhizoids display thin walls with large diameters and are involved in the anchorage to substrate but not in the transport of nutrients or water. The smooth rhizoids have recently been shown to exhibit tip growth similar to that of root hairs (Duckett et al., 2014). Smooth rhizoids are first involved in anchoring spores to the substrate and later as both anchors and absorptive structures similar to root hairs (Duckett et al., 2014, Cao et al., 2014). The gain of these functional rhizoids for the prevention of desiccation, anchorage and potential water and nutrient transport was an essential step in the colonisation of land by the first land plants.

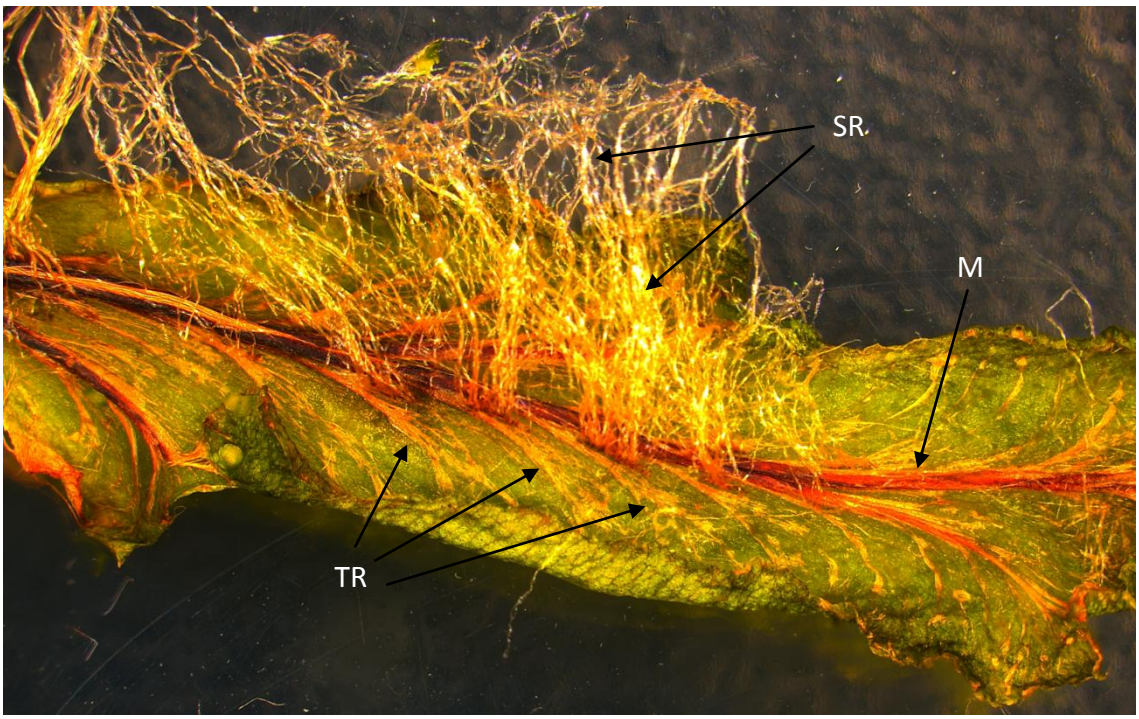



Figure 1.12: Rhizoid structure of *Marchantia polymorpha*. Smooth rhizoids (SR) grow in clusters perpendicular to the thallus axis. Tuberculate rhizoids (TR) grow parallel to the thallus axis and converge on the midrib (M).

Liverworts including *M. polymorpha* are also known to contain oil bodies which contain lipophilic globules suspended in a proteinaceous matrix surrounded by a single unit

membrane (He *et al.*, 2013). The function of these oil bodies has been proposed to be involved in protection from herbivory, desiccation, cold temperature and high light and UV-B stress. The exact function of the oil bodies may not be consistent and their function and structures (Fig. 1.13) in liverwort may depend on the species and its requirements. For instance *Anthelia* liverworts grow in high light and arid conditions yet lack oil bodies suggesting they are not needed for protection against such conditions in this species (He et al., 2013). As oil bodies may contain phenolic compounds they may be important in UV attenuation and protection however this is yet to be determined.

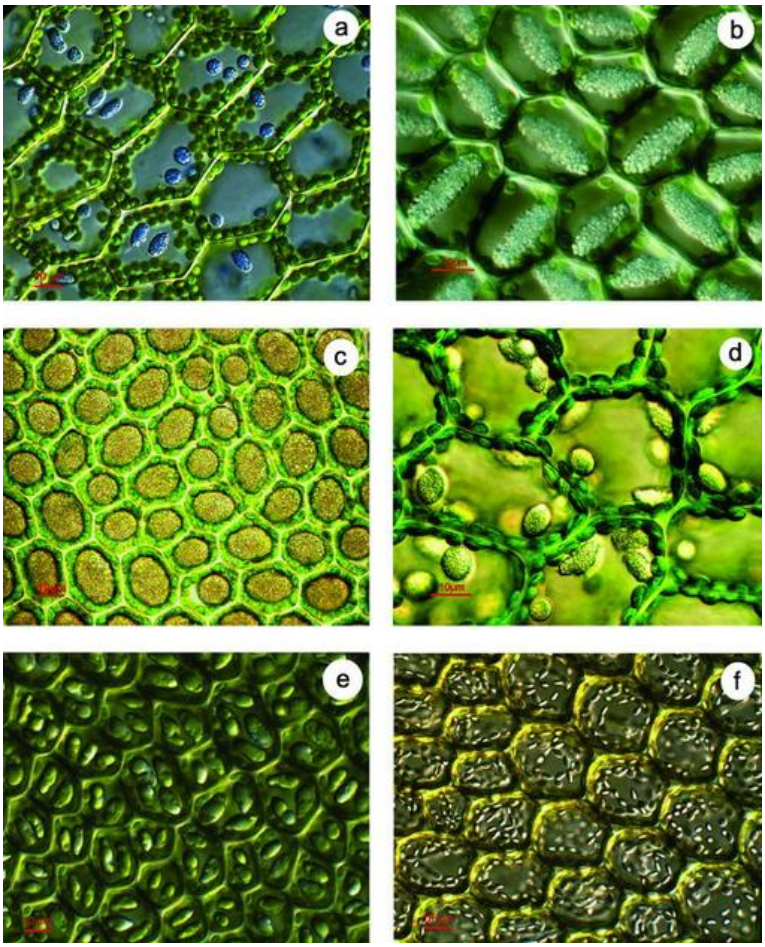



Figure 1.13: Different types of oil bodies in the cells of liverworts: a–b, *Calypogeia*-type; c–d, *Jungermannia*-type; e, *Bazzania*-type; f, *Massula*-type a. *Calypogeia azurea*, b. *Cheilolejeunea anthocarpa*, c. *Radula constricta*, d. *Solenostoma truncatum*, e. *Bazzania tridens*, f. *Trocholejeunea sandvicensis* (From *He et al. (2013)*).

### 1.9.2 Liverworts life cycle

The liverworts dominant life cycle is that of the haploid gametophyte and asexual reproduction, while it forms a diploid state only during sexual reproduction. The sexual cycle of liverworts begins with the formation of the male and female structures usually in response to light and environmental conditions (Benson-Evans, 1964, Chopra & Bhatla, 1983). The male reproductive organs (anthiridium) contain motile gametes while the female organs (archegonium) contain the egg cells. The male gametes require a continuous film of water in order to move to the archegonium and fertilise the egg cells. This is usually mediated through rain droplets that allow transfer of sperm from the antheridial head to the archegonial head and explains one of the reasons liverworts are usually found in moist areas.

Once the egg is fertilised the development of the sporophyte begins within the archegonium. A diploid state is formed within the young sporophyte before the spores develop in the mature sporophyte. The seta which supports the sporophyte is regulated by auxin and elongates to form a stalk which allows the sporophyte to break through the gametophyte tissue to form the mature sporophyte structure (Poli *et al.*, 2003). Once mature the sporophyte ruptures and the spore are released with the help of elators which react to changes in humidity. Once released the spores are able to disperse and upon reaching a suitable area they germinate to form new gametophyte tissue (Fig. 1.14).

Many liverworts including *M. polymorpha* are dioecious (Wyatt & Anderson, 1984) which often results in the male and female gametophytes being largely separated. Raindrop and liquid dispersal of male gamete is limited in such cases so many liverworts have also developed the ability to reproduce asexually. The asexual reproduction of *M. polymorpha* is predominantly via fragmentation of the haploid gametophyte. Simple fragmentation by accidental breakage and dispersal may result in propagation of new plants in surrounding areas. Large distances may be overcome especially where fragmentation occurs near aquatic environments that aid dispersal. *M. polymorpha also* has specialised dispersal structures called gemmae cups. These gemmae cups hold the haploid gemmae which are released into the environment, auspiciously by water droplets that impact the gemmae cup and result in

gemmae being ejected large distances away from the original plant (Brodie, 1951, Shaw *et al.*, 2011, Stieha *et al.*, 2014).

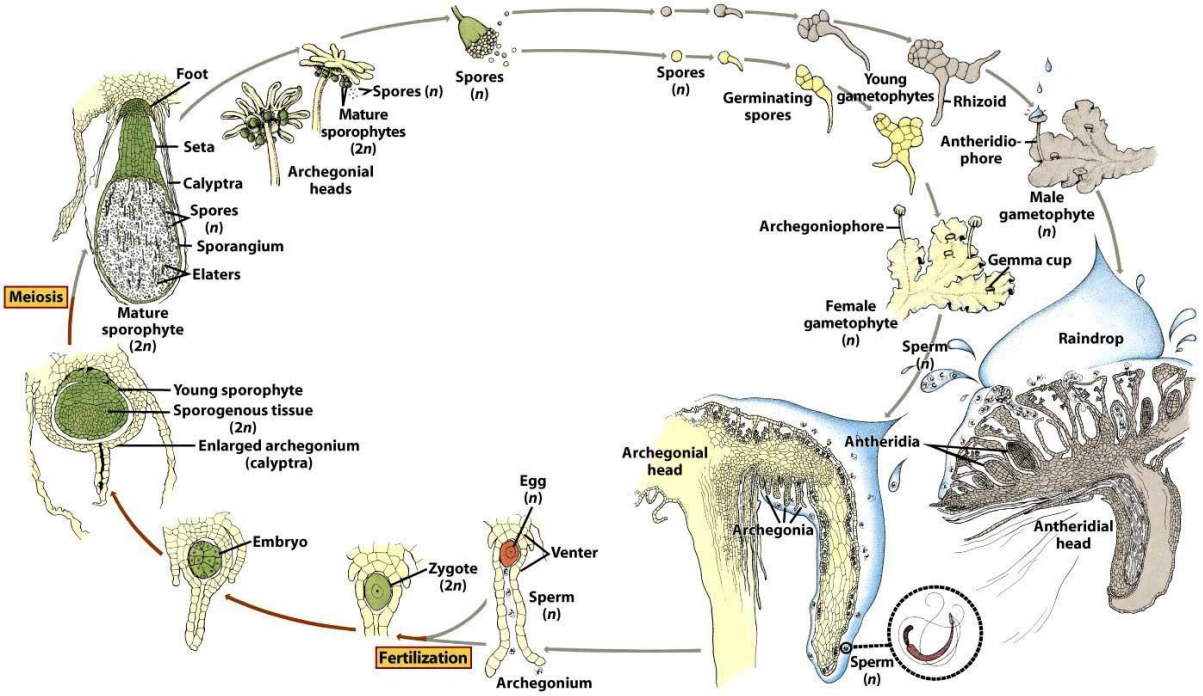



Figure 1.14: Liverwort life cycle (*Marchantia* spp) (From. Raven et al. (2005)).

### 1.9.3 Flavonoid pathway in *Marchantia polymorpha*

Based on the flavonoids that it is known to produce (Markham et al., 1998a, Markham & Porter, 1974), *M. polymorpha* should have genes for at least phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumaroyl-CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerise (CHI), flavanone 3-hydroxylase (F3H), flavone synthase (FNS) and flavanone reductase (FNR) (Fig 1.15). Previous studies on UV tolerance in *M. polymorpha* showed a change in the ratio of the flavones apigenin and luteolin in favour of increased luteolin (Markham et al., 1998a). The study did not use a fixed level of UV-B irradiance in the test group and this may have affected the UV response, especially through utilisation of both

the high and low fluence pathways. Whether this expected flavonoid pathway is utilised in response to UV-B irradiance will be of particular interest in understanding how the first land plants may have been able to deal with the higher levels of incident UV-B as they moved from aquatic environments onto land.

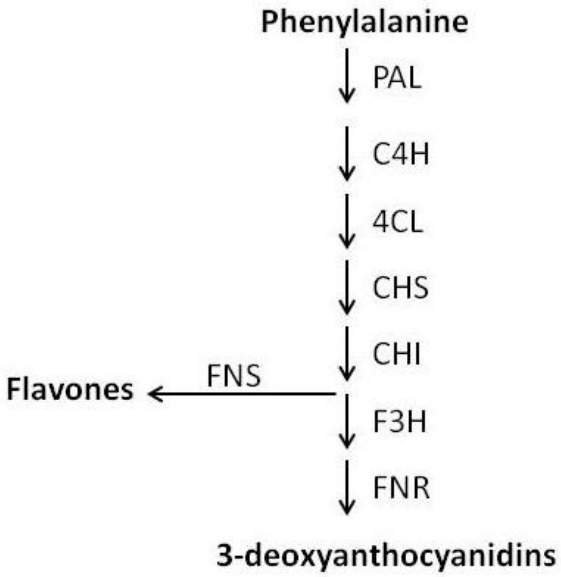



Figure 1.15: Proposed flavonoid pathway of *M. polymorpha*. Starting at phenylalanine enzymatic steps lead to the formation of Flavones and 3-deoxyanthocyanidins. Abbreviations; Phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumaroyl-CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerise (CHI), flavanone 3-hydroxylase (F3H), flavone synthase (FNS), flavanone reductase (FNR).

The objectives for this study were to determine:

1. How *M. polymorpha* responds to UV-B.

2. Whether flavonoid production increases in response to UV-B.

3. If flavonoids are produced, whether flavone production contributes significantly to UV-B protection.

4. How flavonoids localise in *M. polymorpha* thallus tissue upon UV-B irradiance.

5. Whether the production of flavonoids is required for UV-B protection in *M. polymorpha.*

6. What the genetic elements are that contribute to UV-B protection in *M. polymorpha.*

The hypothesis I am testing is that flavonoid production in response to UV-B in *M. polymorpha* will be required for UV-B protection and that this production will be mediated through the conserved UVR8 pathway.

# Chapter 2: Materials and Methods

## 2.1 Controlled Environment Experiments

*Marchantia* plants were grown in a controlled environment that could be supplemented with or without the addition of UV-B radiation in order to test its response and acclimation characteristics. Throughout the study tubs containing *M. polymorpha* were grown under the following conditions.

### 2.1.1 Growth Conditions

Marchantia plants were sourced from the wild at the Plant and Food Palmerston North campus. Marchantia plants were grown on Gamborg B5 medium including vitamins (Duchefa Biochemie) containing 1% phytoagar. Plants were grown in tissue culture tubs with cling film in place of lids. No issues occurred with condensation on lids. Plants were grown for 4 weeks under white light at a PAR of 50 µmol photons $m^{-2}$ $s^{-1}$ with 16 hour photoperiod, before transfer to UV cabinets. Plants were grown for a week in UV cabinets at a PAR of 40 µmol photons $m^{-2}$ $s^{-1}$ and 16 hour photoperiod before UV treatments. PAR was measured by a LI-COR LI-188B Quantum Radiometer (LI-COR Biosciences – Biotechnology, USA). When needed plants were propagated by taking gemmae directly from gemmae cup structures and placed on fresh media. UV fluence was measured using a USB4000-UV-VIS spectrometer (Ocean Optics, USA) between the wavelengths 280-850 nm.

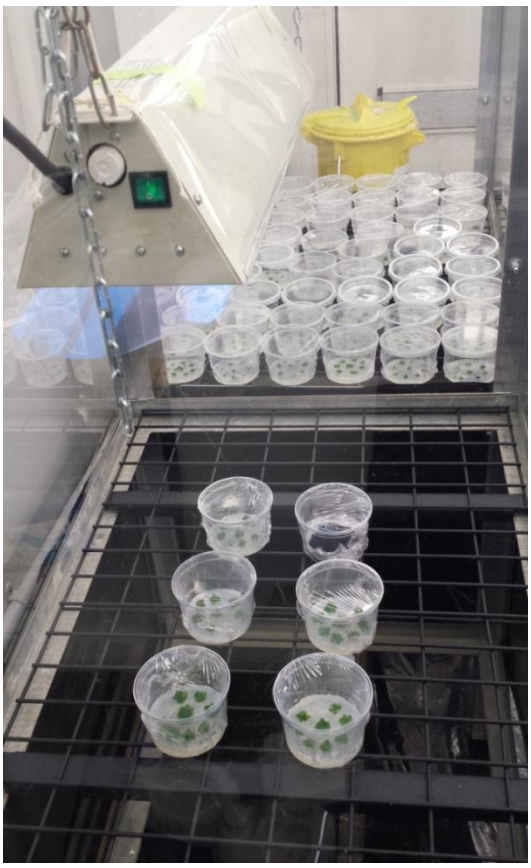


Figure 2.1: Growth cabinets for *M. polymorpha* UV-B testing.

### 2.1.2 UV environment

The relevant fluence for each of the UV-B treatments was 51.05 μW/cm$^2$ under control conditions, 123.93 μW/cm$^2$ for low fluence and 538.24 μW/cm$^2$ for high fluence (Fig 2.2). UV-B was provided by a XX-15M UV Bench Lamp (UVP, Blak-Ray) with a 302 nm broadband fluorescent bulb (UVP, Blak-Ray). Wavelengths below 290 nm were excluded using cellulose acetate film.

Low fluence treated plants received UV-B irradiation for 12 hours in the middle of the 16 hour photoperiod. This continued for 7 consecutive days. High fluence treated plants received UV-B irradiation for 12 hours in the middle of the 16 hour photoperiod on a single day. This exposure did not continue past the first day and plants received normal white light conditions after this exposure.

To determine how plants responded to long term acclimation to UV-B gemmae were plated onto media, allowed one week under light lacking UV-B and were then grown under low fluence conditions for a further 4 weeks before being photographed.

Total overall dosage of low fluence plants equated to 21427 kJ/cm$^2$ at 4 days and 37497 kJ/cm$^2$ at 7 days. High fluence plants received at total dose of 23241 kJ/cm$^2$ on the single day of exposure.

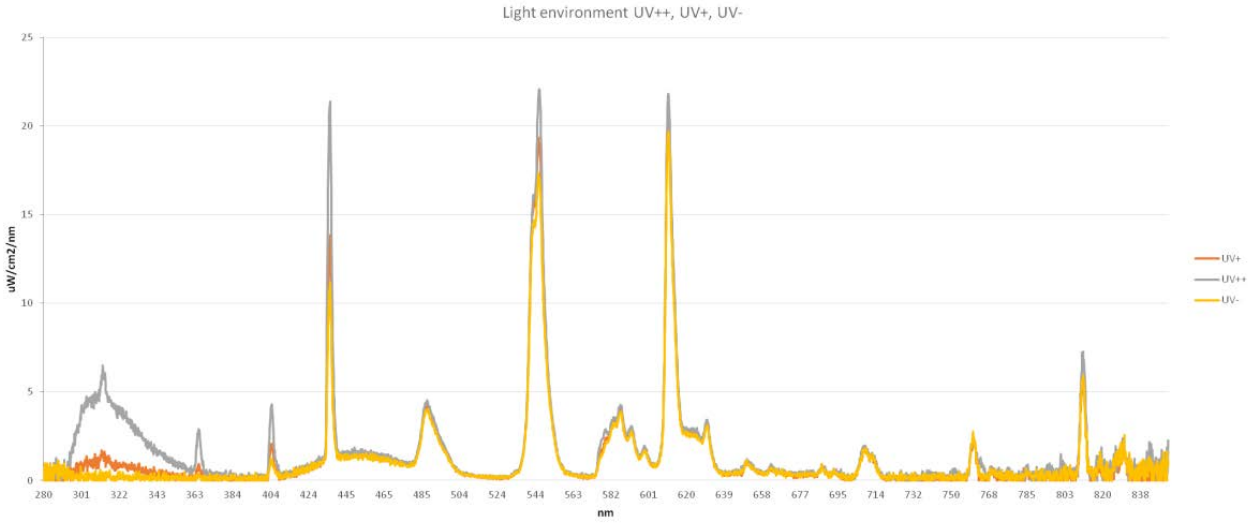



Figure 2.2: Light environment of cabinets. Full light spectrum from 280-850nm is shown for control (UV-), low (UV+) and high (UV++) UV-B conditions.

## 2.2 Biochemical Analysis

### 2.2.1 Methanol extraction

Whole plants were taken and snap frozen in liquid nitrogen. Plants were then freeze dried and 10mg of tissue ground for 30 s at 30 Hz using a Tissue lyser II (Qiagen) to a fine powder. 1 mL of 50% Methanol (v/v Methanol/Water) was added to the ground tissue and this was incubated at room temperature overnight with rotary shaking. Samples were centrifuged at

14,000 rpm for 5 minutes and the supernatant containing flavonoids and UV absorbing compounds collected in a fresh tube.

The supernatant was passed through an Alltech® Extract-Clean™ C18 solid phase extraction column (Grace Davison Discovery Sciences, New Zealand) matched with a Vacuum Manifold (Grace Davison Discovery Sciences, New Zealand). Before loading the supernatant into the C18 SPE column, the column was firstly conditioned by two steps: 3 mL of 100% methanol were added into each column to activate the sorbent ligands; and 3 mL of water was added into each column to equilibrate the sorbent bed. The supernatant of each sample was applied to the SPE device and the flow speed was controlled at 3 mL/min by the Vacuum Manifold. Then the column was washed by 20% methanol, and 2 mL of 100% methanol was supplied into each column to elute the flavonoids from the sorbent bed. The elution through the column were collected in a new 15 mL tube and adjusted with 100% methanol into 3 mL for total UV-absorbing compound analysis. During all of these steps, the column was not allowed to dry until the last elution step was completed.

### 2.2.2 Total UV absorbing Compounds

To determine quantitative changes in total UV-absorbing compounds, absorbance spectrum of the flavonoid extraction between wavelengths 240-750 nm (step width 1 nm) was detected by a Multiskan GO plate reader (Thermo Scientific) with a 96-well micro-plate at 20°C. 100 µL of each sample was transferred onto a 96-well micro-plate for absorbance readings. Total UV absorbing compounds were measured as the total absorbance over 380-420nm per gram of fresh weight tissue.

### 2.2.3 Ultra High Performance Liquid Chromatography (UHPLC)

Flavonoids were extracted from 10 mg freeze-dried ground thallus tissue with 1 mL Methanol: water: formic acid (80:19:1). Ultra High Performance Liquid Chromatography (UHPLC) was used to separate and measure the flavonoids present in extracts of thallus tissue. The UHPLC system used was a Dionex Ultimate® 3000 Rapid Separation LC system equipped with a binary pump (HPR-3400RS), autosampler (WPS-3000RS), column

compartment (TCC-3000RS), and a diode array detector (DAD-3000RS). The analytical column used was a Kinetex XB-C18 50 mm × 3 mm, 2.6 µm (Phenomenex, California, USA), maintained at 35°C. A binary solvent programme was used with Solvent A (formic acid:MQ water, 1:99) and Solvent B (acetonitrile) at a flow of 1 $mLmin^{-1}$. The initial solvent composition was 90%A 10%B until 0.5 minutes, then changed to 50%A 50%B at 2.5 min, and 5%A 95%B at 3.5 min. After a 1 min hold at 5%A 95%B, the composition was returned to 90%A 10%B ready for the next injection. Total UHPLC analysis time was 6 min per sample. All solvent gradients were linear. The injection volume was 3 µL. Spectral data (260–600 nm) were collected for the entire analysis.

Flavones were quantified from chromatograms extracted at 340 nm, and external calibration curves were constructed for luteolin 7-*O*-glucoside (Extrasynthese, Genay, France). Flavone concentrations were calculated as luteolin 7-*O*-glucoside equivalents on a dry weight basis. Individual flavones were identified based on LCMS fragmentation, and comparison to the spectral properties and order of elution reported in Markham and Porter (1974).

### 2.2.4 Total RNA isolation

Whole plants were snap frozen in liquid nitrogen and ground using a mortar and pestle. Ground tissue was then used for RNA extraction. Total RNA was extracted from 100 mg of ground material using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Auckland, New Zealand) according to the manufacturer's instructions. The lysis buffer with 2-β-mercaptoethanol (2-BME) mixture was prepared fresh before use by adding 10 µL of 2-BME to each 1 mL of lysis buffer (final concentration of 2-BME 1% v/v). 700 µL of the lysis solution/2-BME mixture was added to 100 mg of tissue in a 2 mL centrifuge tube and immediately vortexed vigorously for at least 30 sec. The sample was then incubated at 56°C for 3-5 min and centrifuged for 3 min at 14000×g to pellet cellular debris. Without disturbing the pellet, the supernatant was pipetted into a filtration column placed in a 2 mL collection tube and centrifuged at 14,000 ×g for 1 min to remove residual debris. 700 µL of binding solution was added to the clarified solution and mixed immediately with a pipette. Then the solution was transferred with a maximum volume of 700 µL onto a binding column placed in

a 2 mL collection tube. Each portion was centrifuged at 14,000 ×g for 30 sec with the flowthrough discarded after each centrifugation step. The column containing bound RNA was then washed by adding 500 µL of wash solution-1 and centrifuged at 14,000 ×g for 1 min followed by two subsequent wash steps of 500 µL of wash solution-2 and subsequent centrifugation at 14,000 ×g for 1 min. The flow-through was discarded at each step. The column was then centrifuged at 14,000 ×g for 1 min and then transferred to a new 1.5 mL collection tube. The bound total RNA was then eluted with 50 µL of nuclease-free water by application and centrifugation at 14,000 ×g for 1 min. The elution containing purified RNA was stored in a freezer at - 80°C.

### 2.2.5 DNase treatment

Purified RNA samples were treated using the TURBO DNA-free™ Kit (Life Technologies, New Zealand) according to manufacturer's instructions to remove traces of genomic DNA. To the RNA sample, 0.1 volume of 10× Turbo DNase Buffer and 1 µL of TURBO DNase were added, mixed gently by pipette and incubated at 37°C for 30 min. Then 0.1 volume of DNase Inactivation Reagent was added to the reaction, mixed and incubated at room temperature for 5 min, mixing occasionally. The sample was then centrifuged at 10,000 ×g for 1.5 min and the supernatant was transferred to a fresh 1.5 mL tube. The purified RNA was stored at -80°C for further use.

### 2.2.6 Total RNA quantification

The concentration and quality of RNA was determined by spectrophotometric analysis. The absorbance of the sample at 230 nm, 260 nm and 280 nm were measured using a NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Montchanine, USA). The 260/280 nm and 260/230 nm ratios were used to estimate the purity of the isolated RNA. The ratio of absorbance at 260 to 280 nm for purified RNA, calculated by (A260–A320)/(A280–A320) should be between 1.8 and 2.2.

### 2.2.7 cDNA synthesis

cDNA was synthesised using Primescript™ RT reagent kit (perfect real time) (Takara Bio Inc) according to the manual instructions. For each reaction, 300 ng of RNA was added with 2 µL of 5× Primescript Buffer (final concentration 1×), 0.5 µL of Primescript Enzyme Mix I, 0.5 µL of Oligo dT Primer (final concentration 25 pmol) and 0.5 µL of Random 6 mers (final concentration 50 pmol), adjusting with RNase-free water to a final volume of 10 µL. The reaction was incubated at 37°C for 15 min for reverse transcription, and then heated at 85°C for 10 sec to inactivate the reverse transcriptase. The 10 µL reaction was diluted 5 fold and an aliquot of this was taken and diluted a further 5 fold for use in quantitative real time PCR.

### 2.2.8 Quantitative Real Time PCR (qRT-PCR)

For quantitative real time PCR RNA was extracted from thallus tissue and CDNA was synthesised. Primers (Appendix 1) were synthesised for genes of interest to have melting points of $T_m$ = 60°C ( ± 1°C), a minimal secondary structure, unable to form dimmers and amplify a product ranging in size from 80-250 bp for detection by SYBR Green I. Samples for analysis were prepared in 15 µL reactions consisting of 7.5 µL of 2× SYBR® Premix Ex Taq™ PCR Mix (Takara Bio Inc., Norrie Biotech, New Zealand), 0.6 µL of 10 µM primer mix (containing forward and reverse primers), 1.9 µL of nuclease-free water and 5 µL of diluted cDNA template.

Quantitative real-time PCR was performed on an EcoTM Real Time PCR System v4.0 controlled by the EcoTM Software System v4.0 (Illumina, dnature Ltd, New Zealand). Reactions were run in triplicates and the PCR conditions were: 3 min at 95°C for polymerase activation; a cycle of 5 sec at 95°C then 20 sec at 60°C (40 cycles). A melt curve analysis was performed at the end of each run. Analysis was performed using the efficiency corrected comparative CT method (Pfaffl, 2001). Reaction efficiencies were determined for each reaction using LinRegPCR software (Ramakers *et al.*, 2003).

### 2.2.9 Bioanaylser

RNA quality was analysed using an Agilent 2100 bioanlyser. RNA samples that had been DNase treated were used to check RNA quality before being sent for sequencing or nCounter analysis. An RNA nano 6000 kit was used and prepared as per the manufacturers instructions. For each run 1 μl of RNA 6000 nano dye was added to 65 μl of Agilent RNA 6000 Nano gel matrix, vortexed until mixed and spun at 14,000 rpm for 10min. 9 μl of the gel dye mix was then added to the corresponding well in the RNA chip and a plunger used to prime all sample wells with gel. 5 μl of RNA 6000 Nano marker was added to each sample well and the ladder well before 1 μl of sample was added to each sample well. 1 μl of ladder was also added to the ladder well. The chip was then vortexed for 1 minute in an IKA vortex mixer. The chip was then loaded on the Agilent 2100 and analysed using the 2100 expert software. RIN values and gel images were exported and used to demonstrate quality where needed. Between samples the electrodes were washed for 1 min in RNaseZap for 10 s followed by RNase free water for 10 s and left to dry for 10 s before closing the lid or inserting a new chip.

### 2.2.10 Reactive Oxygen Species Fluorescent Reporter

To visualise ROS in the thallus tissue 2',7'-Dichlorofluorescin Diacetate (DCFDA) (Sigma-Aldrich, Auckland, New Zealand) was used. DCFA is a cell-permeable non-fluorescent probe which turns to highly fluorescent 2',7'-dichlorofluorescein upon oxidation. In order to visualise the localisation of ROS in the thallus transverse sections of 1 $cm^2$ whole thallus tissue were incubated in a solution of 50 mM Tris-HCL (pH 7.0) and 200 μM DCFDA. Tissue was incubated for 20 min in the dark before visualisation by on the Eclipse 50i fluorescent microscope (Nikon Instruments Inc) using a GFP filter cube (excitation 488 nm, emission 500-540nm). Images were captured using a Digital Sight DS-U3 (Nikon Instruments Inc) and processed using NIS Elements BR 3.2 (Nikon Instruments Inc).

Quantitative measurement of ROS was performed by snap freezing samples and grinding in liquid nitrogen. 1 mL of chilled TRIS-HCL (pH 7.0) DCFDA solution was added to 10 mg of ground tissue, briefly vortexed and incubated for 20 min in the dark. Samples were

centrifuged at 13,000 rpm for 5 min to pellet debris and 200 µL of supernantant taken and added to a 96 well plate. Plates were analysed using the fluorescent plate reader (FLUOstar Omega, BMG Labtech) with excitation at 480 nm and emission capture at 520 nm. A standard curve of hydrogen peroxide was used to determine the equivalent ROS level of samples.

### 2.2.11 Chlorophyll quantification

Thallus tissue was snap frozen in liquid nitrogen before being freeze dried and ground in mortar and pestle. 10mg of ground tissue was incubated with 80% acetone overnight in the dark and samples were spun for 5 min at 13,000 rpm to pellet cell debris. 200 µL of supernatant was taken and added to a 96 well plate. Absorbance over 240-750 nm (step width 1 nm) was detected by a Multiskan GO plate reader (Thermo Scientific) at 20°C. Chlorophyll content was determine by using the equations for 80% acetone extraction (Sumanta *et al.*, 2014).

### 2.2.12 Dissecting microscopy

Whole plants and thallus tissue was photographed using a dissecting microscope, Olympus SZX16, with camera attachment Olympus DP71. Photos were processed using the microscope software DP controller.

### 2.2.13 Fluorescent microscopy for flavonoid visualisation

To visualise flavonoid localisation the flavonoid specific stain 2-Aminoethyl diphenylborinate (DPBA) was used. Thallus tissue of 1 $cm^2$ was stained for 2 hours in 0.25% (w/v) DPBA and 0.1% (v/v) Triton X-100. Thallus tissue was then visualised for flavonoid fluorescence by fluorescent microscope on the Eclipse 50i fluorescent microscope (Nikon Instruments Inc) using a GFP filter cube (excitation 488 nm). Images were captured using a Digital Sight DS-U3 (Nikon Instruments Inc) and processed using NIS Elements BR 3.2 (Nikon Instruments Inc).

### 2.2.14 RNA sequencing

RNA sequencing was performed on Illumina TruSeq Stranded mRNA libraries by Illumina HiSeq, and was conducted by the Australian Genome Research Facility (Melbourne, Australia) or Macrogen Inc (Seoul, South Korea). Bioinformatics analysis used DEseq2 (Love *et al.*, 2014) with comparison of reads to a de novo transcript assembly, and the published Marchantia genome sequence and transcript assemblies. Bioinformatic analysis was performed by Amali Thrimawithana (Plant and Food Research, New Zealand). Excel spreadsheet were produced that included a *M. polymorpha* ID (MpID), baseMean, log2foldchange, pValue, Adjusted pValue (padj), enzyme commission description (ECDesc), gene ontology description (GODESC), kyoto encyclopedia of genes and genomes orthology description (KEGGORTHDESC), eukaryotic orthologous groups ID (KOGID), eukaryotic orthologous groups description (KOGDESC), protein analysis through evolutionary relationships description (PANTHERDESC) and protein family description (PFAMDESC). Analysis and comparison of individual gene transcription was performed using data obtained from these excel spreadsheets.

### 2.2.15 nCounter Analysis

nCounter services were provided by New Zealand Genomics Limited (Dunedin, New Zealand) in partnership with Otago Genomics Bioinformatics Facility (Dunedin, New Zealand). Total RNA of 100 ng in 5 uL total volume was processed by using the standard nCounter total RNA protocol. The total RNA and codeset were combined with hybridisation buffer and incubated at 65°C for 21 hours. The codeset consists of reporter and capture probes that hybridise to the target sequences of interest, forming a tripartite complex. Hybridised samples were processed in batches of 12 by using the robotic prep station (High Sensitivity Protocol, 3 hours per 12-sample cartridge). Data acquisition was performed by using the GEN2 Digital Analyzer (DA), with the "Max" Field of View (FOV) setting (555 images per sample; 5 hour scan per cartridge). Raw data was exported as RCC files from the DA, and QC checked by using NanoString's nSolver data analysis tool. Normalised counts used in analysis contained background-corrected counts (mean + 1SD) normalised by nSolver to the geometric mean of both the positive controls (between lane hyb effects) and all nominated reference, a.k.a housekeeping, genes (RNA input effects) for all samples in a single table.

### 2.2.16 Statistics

Statistical analyses were conducted using the Minitab 16 statistical software package (Minitab Inc., UK). Changes in the total absorbing compounds, flavone levels and transcript abundance differences of *M. polymorpha* plants were analysed by One-way analysis of variance (ANOVA). UV-B absorbing and flavone amounts were analysed and reported using One-way ANOVA with Fishers's least significant difference (LSD) test at 5% and 1% levels. Transcript abundance differences were analysed by One-way ANOVA using Dunnett's test at 5%. WT was used as the control group for comparison of means against *hy5*, *rup1* and 35S:MYB14 plants. Three replicates were taken for each treatment or time point and comprised of independent whole plants. RNA-sequencing and nCounter analysis used three replicates consisting of 5 whole plants each.

# Chapter 3: The physiological response of *M. polymorpha* to UV-B irradiance

## 3.1 Introduction

Plants not only respond to UV-B through the production of secondary metabolites but also through some distinct morphological changes (Jordan, 2017). In general, plants may exhibit a compact growth structure with lower leaf areas and increases in leaf thickness (Robson *et al.*, 2015, Jansen *et al.*, 2017 "In Press"). A long term study of *M. polymorpha* under varying UV-B conditions showed a decreased ground coverage of thallus tissue and the number of gemmae cups were decreased under UV-B conditions, as compared to ambient light and controls lacking UV-B (Markham et al., 1998a). The authors noted that these changes were likely due to the increased stress exhibited by the addition of UV-B. Studies of the Antarctic leafy liverwort *Cephaloziella varians* found that under high UV-B conditions plants exhibited a decrease in chlorophyll content as compared to those plants under low UV-B fluence, which were noticeably greener (Newsham *et al.*, 2005). The moss *Physcomitrella patens,* which is a basal land plant, has been studied for its morphological characteristics under UV-B conditions (Wolf *et al.*, 2010). Gametophores grown for 5 weeks under supplemented UV-B showed a decrease in chlorophyll content as compared to that of the UV-B lacking control. Gametophore colony size was also significantly reduced in UV-B treatments. High UV-B conditions also inhibited the germination of spores while low UV-B allowed spores to grow (Wolf et al., 2010).

High UV-B conditions can induce plant damage that is apparent through distinct glazing and bronzing of the leaf surface. Glazing and bronzing surface phenotypes have largely been attributed to the oxidisation of phenolic compounds and represents a common phenotype in UV-B damaged plants (Cen & Bornman, 1990, Green & Fluhr, 1995, Ambler *et al.*, 1975). In *P. patens* under high UV-B conditions, bleaching and necrotic legions are apparent but appearance of bleaching can be delayed by 2 weeks from the initial irradiation event (Wolf et al., 2010).

ROS signalling is also an important part of the UV-B response and helps show the differentiation between damaging UV-B fluence that may create 'distress' as compared to low fluence UV-B which may cause 'eustress'. This difference may largely be mediated by the ROS burden on the plant (Hideg et al., 2013), so the measurement of ROS would be beneficial in understanding the response of *M. polymorpha* to both high and low UV-B.

To understand how *M. polymorpha* responds to UV-B, plants were exposed to three different treatment conditions that corresponded to high, low and no UV-B fluence. Plants were analysed to understand the response of *M. polymorpha* to such conditions.

## 3.2 Results

### 3.2.1 *M. polymorpha* response to high and low UV-B fluence

Plants were exposed to both high and low fluence UV-B to determine the effect on plant response. High fluence treatment corresponded to a UV-B fluence of 538 $\mu W/cm^2$, while low fluence corresponded to 123 $\mu W/cm^2$. Low fluence treatment consisted of multiple 12 hour exposures in the middle of the photoperiod while high fluence treatment used only one UV-B exposure. An untreated control was used that did not have any added UV-B. Plants under high fluence treatment showed slight bronzing of the thallus tissue (Fig. 3.1). Low fluence treatments did not show increased bronzing when compared to untreated thallus, although a darkening of the thallus tissue was observed (Fig. 3.1). Dissecting micrographs of the thallus tissues under higher magnification showed changes to surface structure as well as greening in tissues of the thallus under the different treatments (Fig. 3.2). A darkening of the tissue, especially around the areolae was observed in the low fluence treated thallus which was not observed in the control or high fluence plants. High fluence thallus showed a loss of greening and disruption of the normal thallus surface structure resulting in increased surface reflectance (Fig. 3.2). This surface reflectance was particularly pronounced in areas that were directly affected by UV-B radiation and may indicate a protective mechanism. This same reflectance was not seen in low fluence treatments (Fig. 3.3).

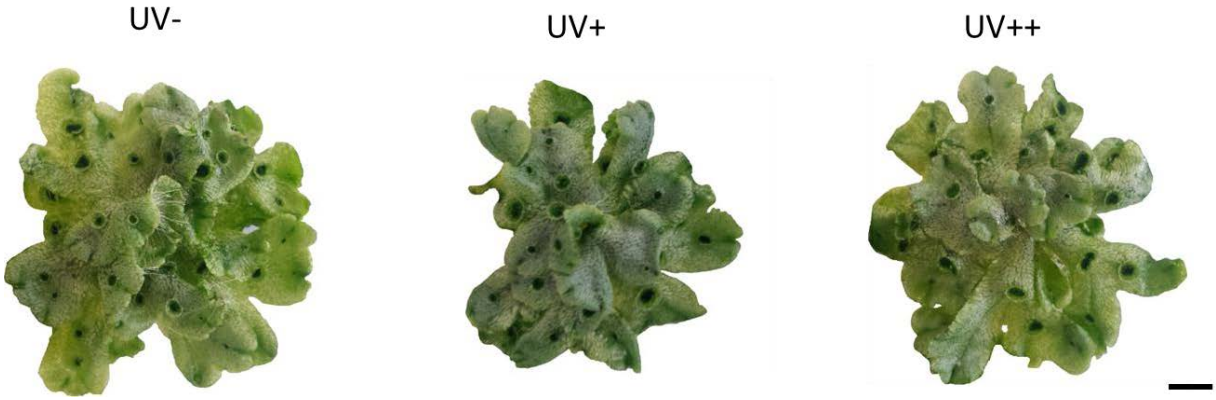



Figure 3.1: Whole plant response to UV-B light treatments. Thallus tissue showing response to white light without UV-B (UV-), addition of low fluence UV-B (UV+) and high fluence UV-B (UV++). Images taken 3 days post treatment. Scale bar = 1cm

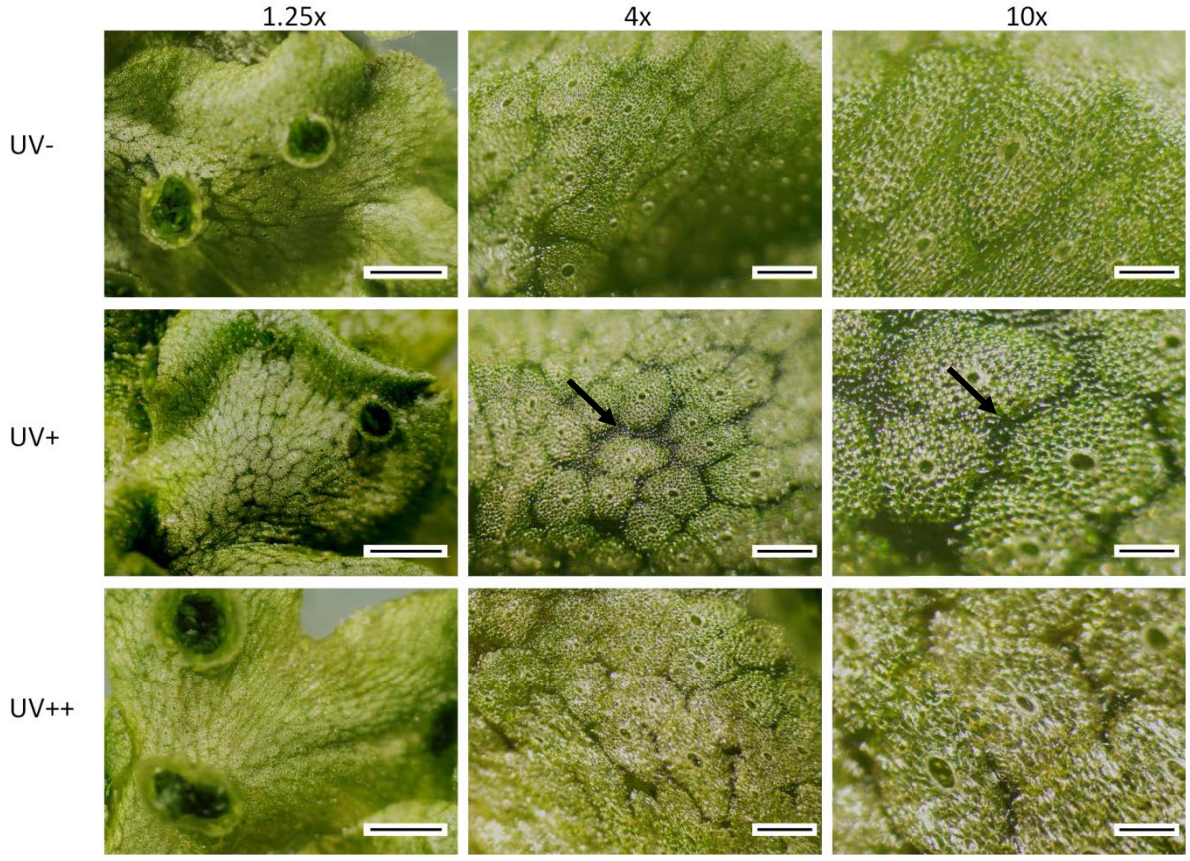



Figure 3.2: Dissecting micrographs of thallus tissue under UV-B treatment conditions. UV+ treated thallus shows darker tissue around areolae (Arrow). Images taken 7 days post treatment. Scale bars = 200 μm (1.25x), 50 μm (4x), 20 μm (10x)

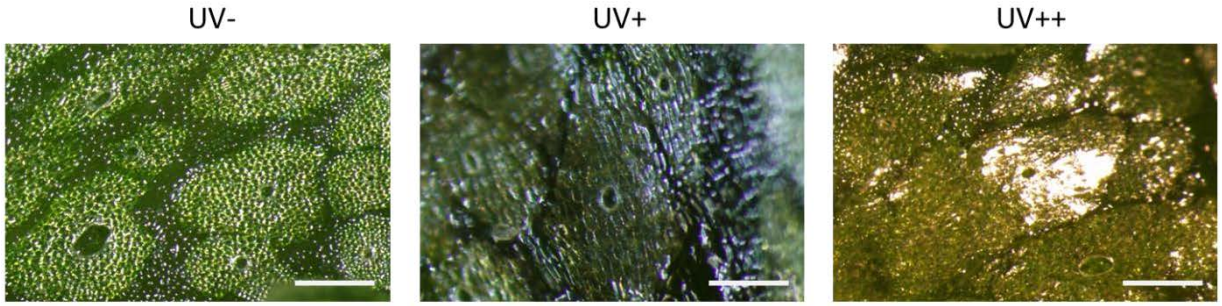



Figure 3.3: Surface reflectance changes in thallus tissue under UV-B treatments. Control (UV-), low (UV+) and high (UV++) fluence treatments are shown. Images taken 7 days post treatment. Scale bars = 20 μm

The darkening of thallus tissue and loss of greening in the high fluence treatment was not attributed to the loss, or gain of chlorophyll content, and no significant differences in chlorophyll content were observed under each of the treatments (Fig. 3.4). A change in the ratio between α and β chlorophyll content was not observed under any of the treatment conditions (Data not shown).

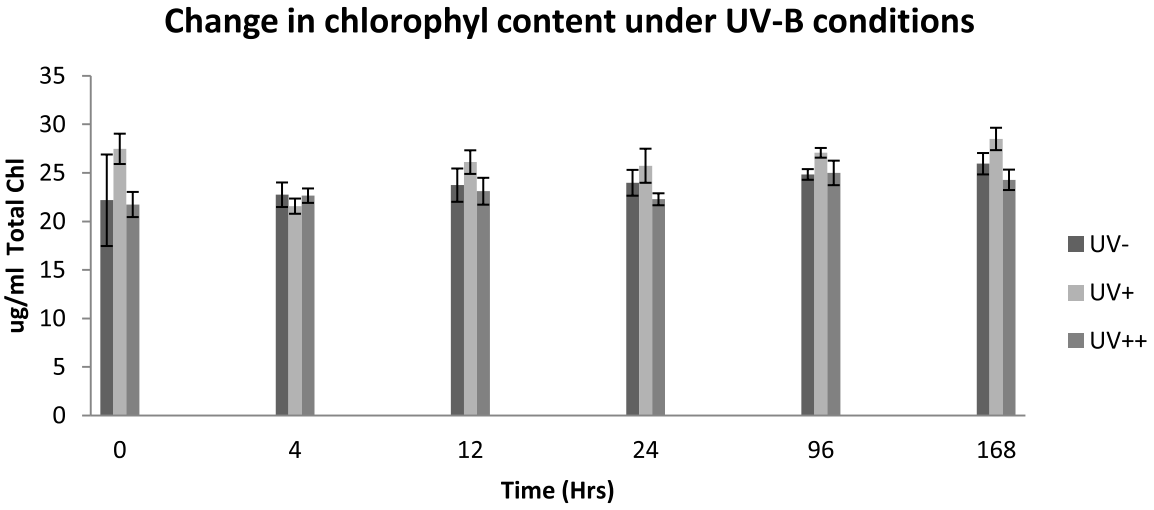



Figure 3.4: Change in chlorophyll content of whole plants over time under UV-B treatment. Data show total chlorophyll content α + β μg/ml dry weight tissue. Means are plotted for each time point and scale bars represent the standard error (n=3).

UV-B is known to affect plant tissue through the production of ROS so thallus tissue was sampled to determine the contribution of ROS production under high and low fluence. ROS were measured by both fluorescent microscopy and quantitatively using the same fluorescent probe. ROS production was enhanced in the chloroplasts of plants that received high fluence UV-B (Fig. 3.5). This ROS production was not seen in the untreated or low fluence treated thallus tissues (Fig. 3.5). All treatments showed ROS production between the areolae (Fig 3.6). Quantitative measurement of ROS production showed an increase in ROS at 12 hours post treatment within high fluence treated thallus (Fig. 3.7). No significant differences were seen in the non-treated or low fluence treated thallus (Fig. 3.7).

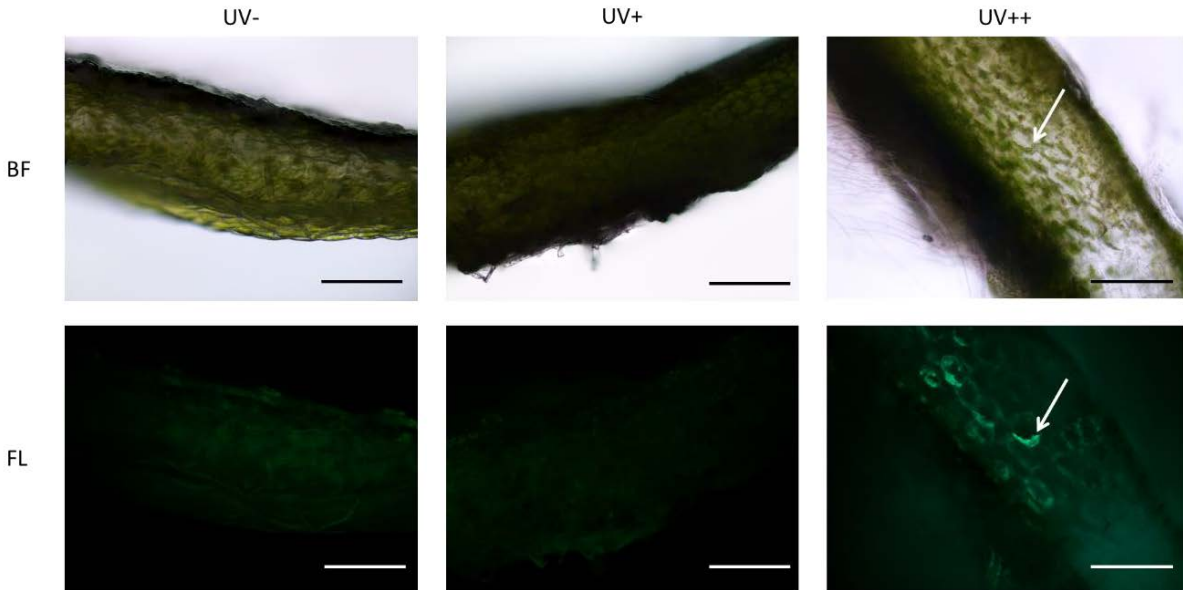



Figure 3.5: Visualisation of ROS production in UV++ treated thallus. Fluorescent (FL) and Bright-field (BF) micrographs of transverse sections of thallus tissue stained with 2′,7′-Dichlorofluorescin diacetate. Bright green fluorescence indicates large amounts of ROS production in chloroplasts of high fluence thallus (white arrows). Images were taken 12 hours after UV-B treatment. Scale bar = 100μm

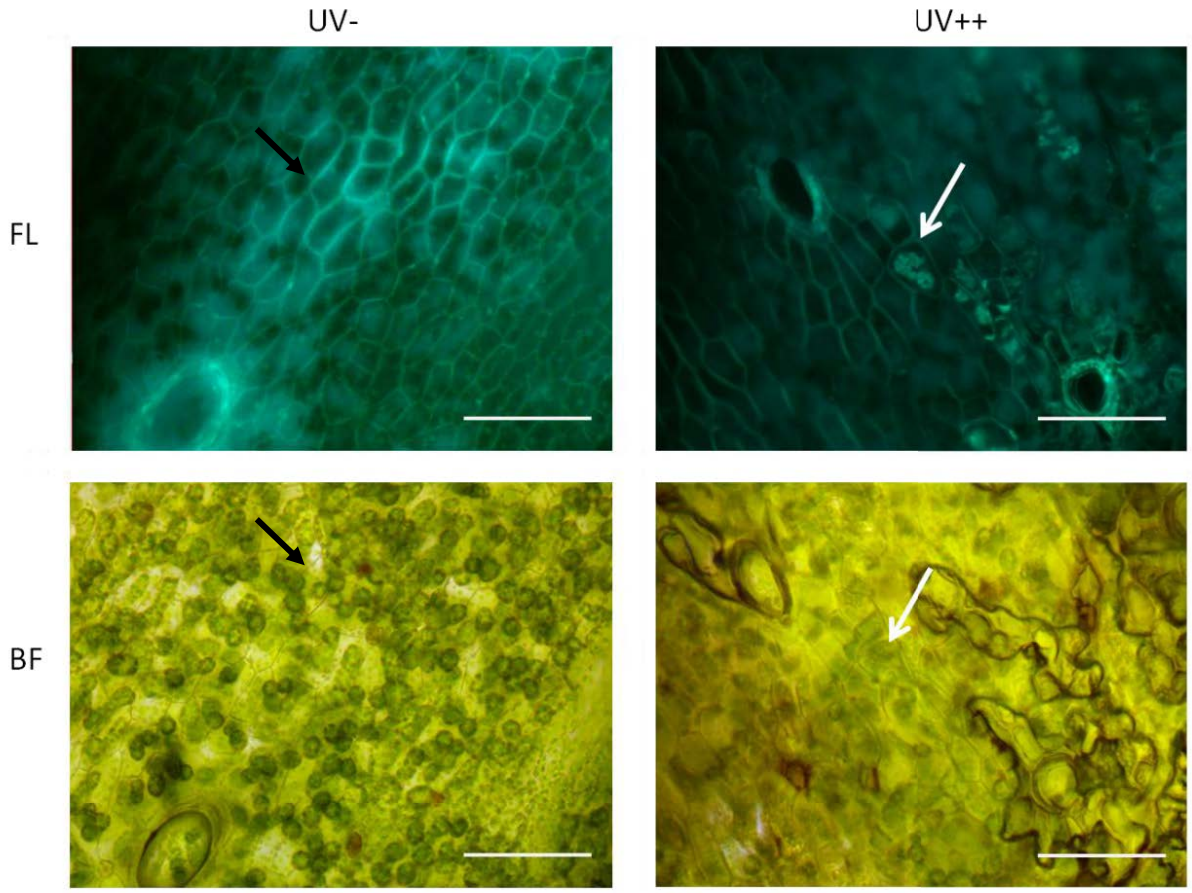



Figure 3.6: ROS production in high fluence treated thallus tissue. Fluorescent (FL) and Bright-field (BF) micrographs of thallus tissue stained with 2',7'-Dichlorofluorescin diacetate which fluoresces green upon oxidation by ROS. UV- thallus indicates ROS between the areolae regions (black arrows). UV++ treated thallus tissue shows high levels of ROS production with chloroplasts (white arrows). Scale bar = 100 μm. Images were taken 12 hours after UV-B treatment.

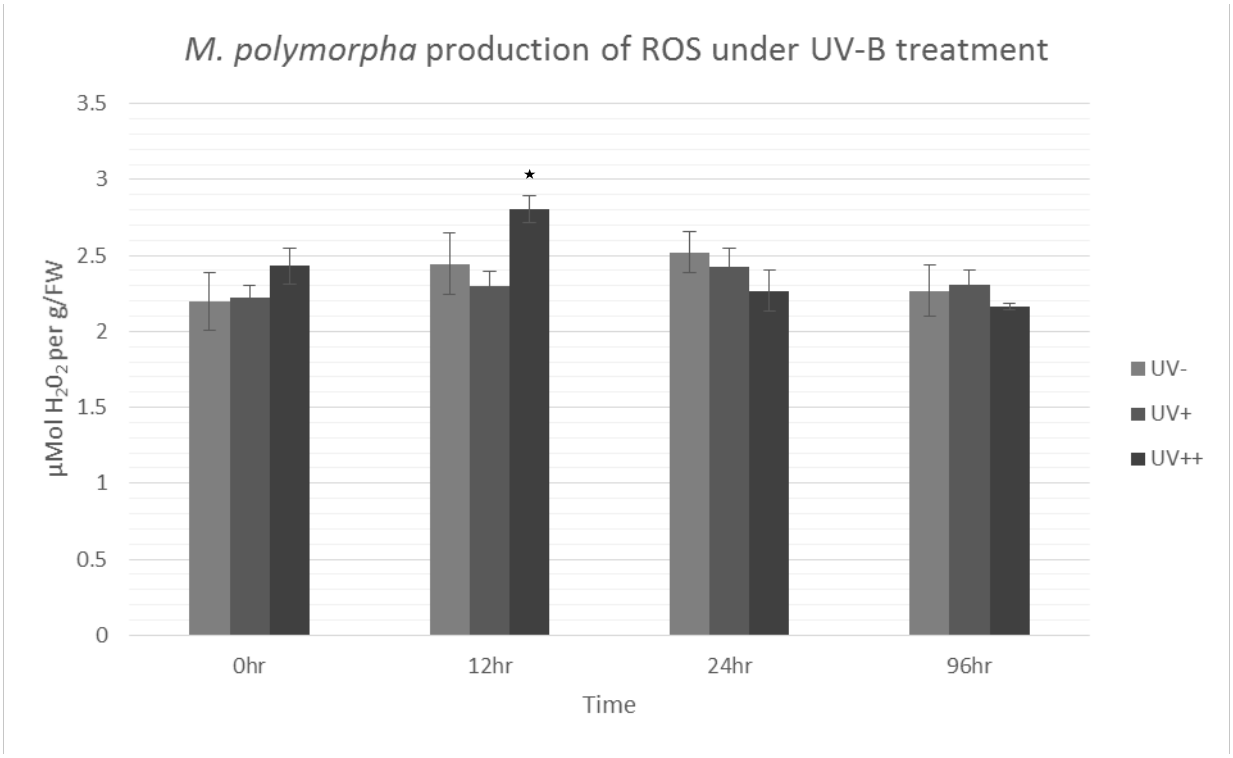



Figure 3.7: Production of ROS by *M. polymorpha* under different UV-B treatments. ROS production was estimated using the fluorescent marker 2',7'-Dichlorofluorescin diacetate (DCFA). Sample fluorescence was compared to a standard curve of $H_20_2$ to estimate total ROS as $H_20_2$ equivalent in thallus tissue. Significant difference from WT within UV-B treatment groups is shown (*$P$<0.05). Error bars = Standard error (n=3)

## 3.3 Discussion

*M. polymorpha* was shown to respond to both high and low UV-B fluence through changes in thallus morphology. In particular high fluence had the largest effect with increases in the surface reflectance and bronzing of the thallus surface while low fluence exhibited no bronzing but a darkening of the thallus. The bronzing and reflectance seen in high fluence treated plants is typical of a high fluence response and damage that is seen in other studies using damaging high fluence UV-B (Cen & Bornman, 1990, Green & Fluhr, 1995). Interestingly the high fluence treated thallus also showed a large increase in the reflectance characteristics (Fig. 3.3). Reflectance from the leaf surface is known to help protect some plants against high irradiance and UV-B wavelengths (Holmes & Keiller, 2002, Caldwell *et al.*, 1983, Johnson, 1975). However, UV-B reflectance from the leaf surface has been proposed to be low in many species except those having densely pubescent or 'woolly' leaves (Holmes & Keiller, 2002). Removal of cuticle wax from the succulent plant *Cotyledon orbiculata,* which has high normal reflectance, resulted in a decrease in the $F_v/F_m$ measure of photosynthetic capacity and the waxy cuticle was thought to have a photoprotective function (Robinson *et al.*, 1993). While *M. polymorpha* in this study showed an increase in surface reflectance in high UV-B treatments, the changes in the cuticle were not measured and changes were based on a visual perspective. Increases in surface reflectance affect broad ranges of wavelengths (Holmes & Keiller, 2002) not just that of UV-B or visible light. It may be that increased reflectance in the surface may protect against or help reduce light irradiance from a broad range of wavelengths in order to reduce further damage to already compromised photosynthetic systems. *M. polymorpha* that was subject to high UV-B irradiance exhibited this surface reflectance as well as surface bronzing but thallus tissue did not senesce. Thallus tissue was able to recover after the high UV-B treatment which may be helped by this increased reflectance reducing further potential photodamage from ambient light, which may include UV-B, during the recovery period. However while UV-B has been shown to increase wax formation the protective attributes may be largely due to the increased flavonoid and UV-B absorbing compounds that may be contained within them rather than the increase in reflectance that results from increases in wax formation (Holmes & Keiller, 2002, Gonzalez *et al.*, 1996, Cen & Bornman, 1993).

Studies in UV-B treated plants have shown changes in the total chlorophyll and the Chl α/β ratio (Smith *et al.*, 2000, Jordan *et al.*, 1994). Measurement of the chlorophyll level and Chl α/β ratio in this study showed that no change in the total chlorophyll or ratio occurred after any of the treatment conditions. This was measured until 7 days after treatments had first begun and no trend was seen either to suggest that longer term loss or gain may occur (Fig. 3.4). In other plants the effect of UV-B on chlorophyll content and ratio is varied, with some species such as pea showing both decreased total chlorophyll and a reduction in the Chl α/β ratio (Jordan et al., 1994), while others have little to no change (Caldwell *et al.*, 1982, Smith et al., 2000). The bryophytes have also been studied for their change in chlorophyll level with varying UV-B (Boelen *et al.*, 2006). Total chlorophyll levels were found to be unchanged by UV-B in a variety of bryophytes studied in the Antarctic region (Huiskes *et al.*, 2001, Lud *et al.*, 2003, Newsham, 2003). However, it must be noted that although these studies looked at plants within the bryophyte family of which *M. polymorpha* belongs, the environment in which the studies were conducted may have greatly influenced the result. The apparent protection of the chlorophyll in *M. polymorpha* may be due to the production of UV-B screening and antioxidant flavonoids that are produced in response to UV-B as discussed in Chapter 4.

While chlorophyll levels remained unchanged we still observe differences in the greening phenotype of thallus tissue when treated with UV-B. In particular the low fluence treatment resulted in an increase in greening of the thallus tissue which was not due to changes in chlorophyll content. This increased greening of plant tissue has been previously observed in higher plants that showed increased levels of phenolics and flavonoid compounds (Bieza & Lois, 2001). This increase also resulted in tolerance to UV-B radiation. In our study the greening of thallus tissue may be due to an increase in flavonoid compounds in response to UV-B for protection. The measurement of increases in flavonoid levels is reported in chapter 4. Such increases would explain the darkening phenotype we observe in low fluence plants without a change in chlorophyll level.

*M. polymorpha* was also seen to have a significant increase in the ROS under high UV-B fluence treatment as compared to the low treatment and control lacking UV-B (Fig. 3.7). Such increases in ROS production in response to UV-B are not uncommon in plants (Hideg et al., 2013). This increase in ROS coincided with the time point where plants had been under

the high UV-B treatment for the longest amount of time and it is understandable that the ROS burden was at the highest in this time point. Low UV-B treatment did not show the same oxidative burst and the level of ROS in the low UV-B treated plants showed no significant change. It may be that ROS in low fluence treated plants are dealt with by ROS scavenging compounds, such as flavonoids, before they build up to detectably higher levels. ROS in high fluence plants may increase to levels that are unable to be dealt with by the normal ROS scavenging mechanisms, and as such we see the ROS increase at 12 hours. It must be noted that the high UV-B treatment environmental conditions used in this study may not truly reflect that of the natural environment for which *M. polymorpha* grows. The PAR in our study is relatively low while the UV-B level is very high under high fluence conditions, at an approximate conversion to 13 on the UVI index. UV treatment with low background PAR is known to result in increased UV stress. In New Zealand on the highest UV day we can expect a value of around UVI 12, which also comes with a very high PAR value. Correspondingly the UV-B stress dealt to high fluence treated plants would be the equivalent of removing them from a shaded environment and placing them in direct sunlight at solar noon, without desiccation or temperature affects. This may not occur naturally outside of controlled environments, so the ROS burst we see here may be a general stress and damage response rather than a specific UV-B acclimation response. For the study of how *M. polymorpha* may respond to UV-B, such high damage and ROS production may be useful in determining contribution of ROS scavengers such as flavonoids as described in further chapters. Nevertheless under more normal conditions such as the low UV-B treatment, ROS may act as an important signalling molecule in the acclimatisation response (Hideg et al., 2013).

ROS production was seen to be localised in high fluence plants in the chloroplasts (Fig. 3.6). Chloroplast contain the photosynthetic machinery which is particularly vulnerable to UV-B damage and the ROS production seen may indicate that ROS are being produced as a result of this damage. Interestingly although ROS was seen to accumulate within the chloroplasts no change in the chlorophyll level was observed under high fluence conditions (Fig. 3.4). High fluence UV-B only occurred for a single 12 hour period, which was enough to cause visible changes to the thallus and the ROS accumulation we see here. A single treatment able to cause such change may then be diminished over time by ROS scavengers or secondary metabolite induction. However sustained UV-B irradiance over multiple days at high fluence

levels may have been enough to overwhelm any protective response and cause a loss in chlorophyll levels and further thallus damage and possibly whole plant death. The same localisation of ROS was not seen in low fluence plants which did not differ from that of the untreated control. Interestingly some ROS localisation was seen to naturally occur in plants between areolae on the thallus (Fig. 3.6). These regions would be considered to have the least benefit of any protective UV-B screening compounds that may be contained in the epidermal cells and thus may be more vulnerable to incident UV-B irradiance, similar to that of UV-B penetration through the anticlinal cell walls of other plants (Jordan, 1996). *M. polymorpha* also has large air pores to allow gas exchange that cannot close, and may allow penetration of UV-B inside the thallus.

Overall *M. polymorpha* responds to UV-B light at both high and low fluences, with high fluence UV-B showing signs of damage to thallus tissue and increased ROS production, especially within the chloroplasts. Such damage is not uncommon in high UV-B studies and while damage was not seen in low fluence UV-B plants, this low level UV-B is more relatable to natural environmental conditions and may result in the acclimation response through non-visual changes in secondary metabolites such as flavonoids.

# Chapter 4

# *M. polymorpha* responds to UV-B through the production of UV absorbing compounds that include flavonoids

## 4.1 Introduction

A large amount of the UV-B response by higher plants is through the production of flavonoid compounds to mitigate damage either through the screening of wavelengths in the UV-B range or through the absorption of damaging ROS (Jansen et al., 1998). However studies in *M. polymorpha* revealed that although a change in the ratio of luteolin to apigenin occurred under UV-B conditions the level of flavonoids that were found did not increase significantly under increasing UV-B irradiance (Markham et al., 1998a). Liverworts are proposed as having characteristics of the first land plants, and as such the ability to deal with UV-B radiation would have been important. Unlike the green algae they are thought to have arisen from, they have the ability to produce flavonoid compounds and the acquisition of this ability is thought to have been important for this first step onto land. While *M. polymorpha* has been shown to produce flavonoids we looked to determine how the production of flavonoid compounds may relate to both high and low fluence UV-B irradiance and whether the ratio of apigenin to luteolin changed under such conditions. High fluence irradiation may induce non-specific changes while the low fluence treatment may cause specific UV-B induction of the flavonoid pathway. Using controlled environment cabinets we were able to treat *M. polymorpha* at different UV-B fluence and measure the production of total UV absorbing compounds, total flavonoid compounds and their localisation in thallus tissue.

## 4.2 Results

### 4.2.1 *M. polymorpha* production of UV-B absorbing compounds

To determine if *M. polymorpha* would respond similarly to higher plants to UV-B through the production of UV-B absorbing compounds, thallus was treated and the total UV-B absorbing compounds measured under each treatment condition over time. Total absorbing compounds are those extracted from whole thallus tissue by methanol that absorbed within the 280-320 nm range. Under control conditions no statistically significant change was seen in the amount of UV-B absorbing compounds over time. Low fluence treated thallus showed significant increases after 12 hours of treatment while the high fluence treated plants show some small increases, but these were not statistically significant. Low fluence treated plants continued to produce absorbing compounds until a peak was reached at 96 hrs after which accumulation levelled off (Fig. 4.1).

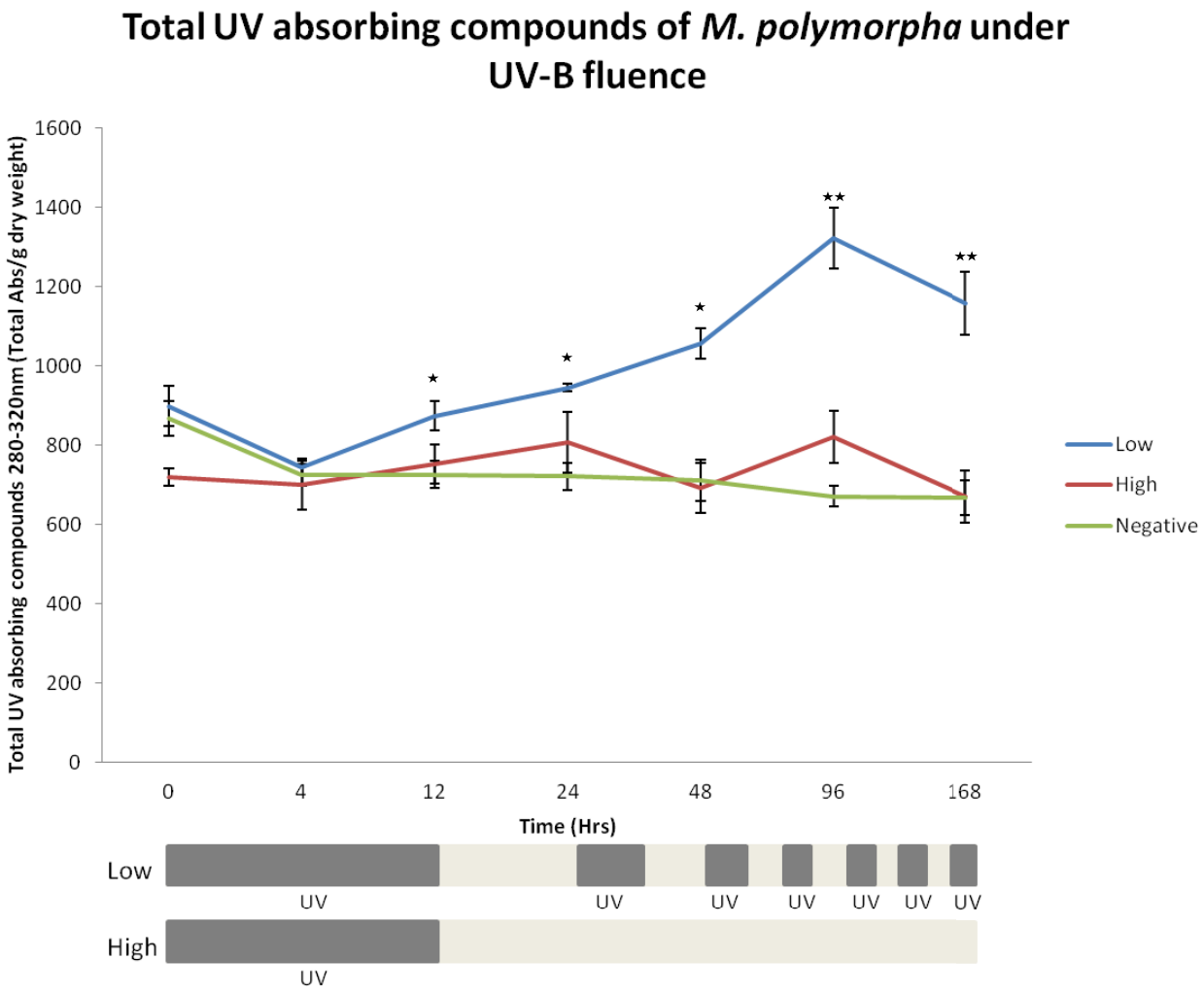



Figure 4.1: Total UV-B absorbing compounds of *M. polymorpha* under UV-B fluence. Total UV absorbing compounds were measured after extraction from thallus tissue at varying times from the beginning of UV treatment. Shaded bars below the graph indicate the hours where the plants were under UV-B irradiation (UV). Untreated, High (UV++) and low (UV+) fluence treatments were measured. (*$P$<0.05, **$P$<0.01). Error bars = Standard Error (n=3)

### 4.2.2 Flavones contribute to the change in UV-B absorbing compounds

To determine if the increase in UV absorbing compounds was due to increase in the flavone content, samples were run through UPLC and the total, apigenin-based and luteolin-based flavones were quantified. Apigenin-based flavones were those comprising of apigenin 7 mono-*O*-glucuronide. Luteolin-based flavones were those of the combined levels of luteolin 7,4' di-*O*-glucuronide, Luteolin-di 7,3'di-*O*-glucuronide and Luteolin 4'-mono-*O*- glucuronide.

Control thallus showed no increase in the amount of total, apigenin or luteolin flavones. Total flavones were seen to increase in both the low and high fluence treated plants (Fig. 4.2). Low fluence treated plants showed an increase from approximately 1250 μg/g to over 2500 μg/g after 96 hours of treatment. Large variation was observed in the 24 hour sample (Fig. 4.2). High fluence treated plants showed a significant increase in total flavones after 24 hours. Total flavones rose from an initial level of around 1000 μg/g to a peak of 1500 μg/g at 96 hrs (Fig. 4.2).

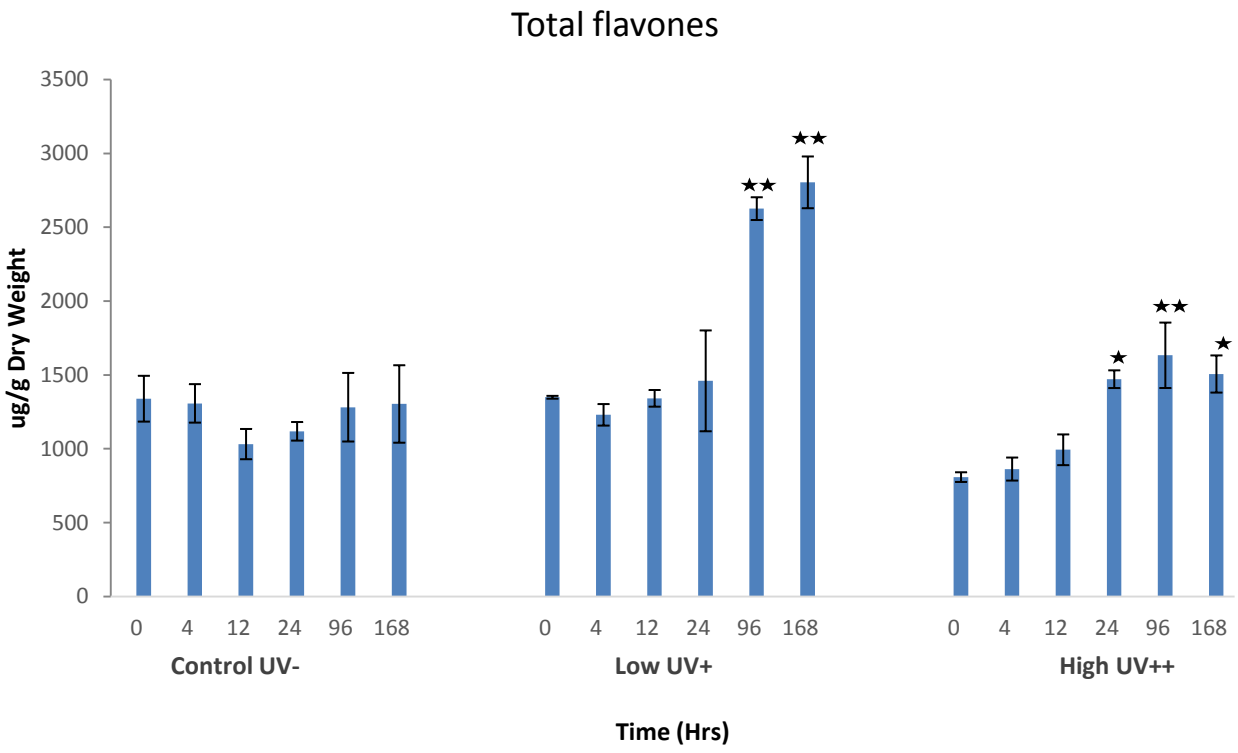



Figure 4.2: Total flavone production of *M. polymorpha* under UV-B treatment. Total flavone amounts were measured by UPLC and determined on a μg/g dry weight basis. (**P*<0.05, ***P*<0.01). Error bars = Standard error (n=3)

Apigenin-based flavones were measured and found to increase significantly in the low fluence treated plants after 96 hrs. Amounts increased from 600 μg/g to over 1200 μg/g. High fluence treated plants showed significant increases after 24 hrs with a change from

approximately 400 μg/g to 800 μg/g. No significant changes were seen in untreated thallus tissue (Fig. 4.3).

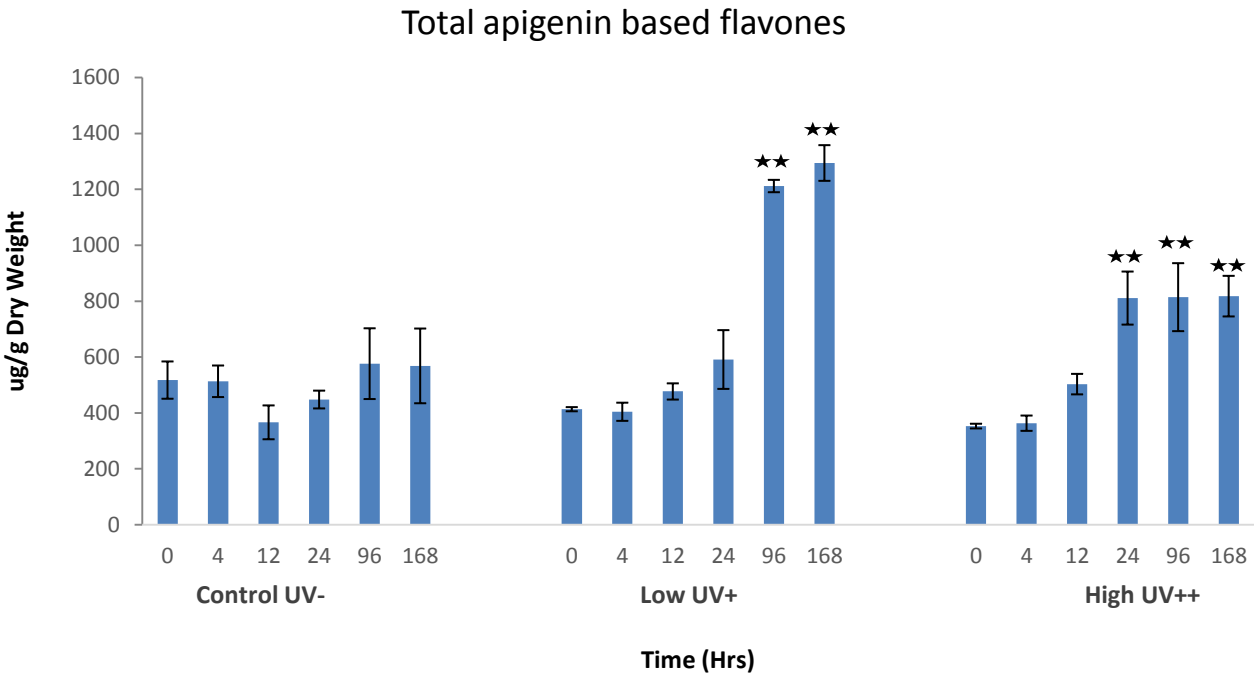



Figure 4.3: Total apigenin-based flavones produced by *M. polymorpha* under UV-B treatment. Total apigenin flavones were measured by UPLC for treated thallus tissue. (**P*<0.05, ***P*<0.01). Error bars = Standard error (n=3)

Luteolin-based flavones were also measured and found to increase in low fluence treated tissue after 96 hours. Amounts rose from 800 μg/g to over 1400 μg/g. High fluence treated tissue showed a significant increase at 96 hours only, which corresponded to an increase from around 500 μg/g to 800 μg/g. No significant changes were seen in the untreated samples over time (Fig. 4.4).

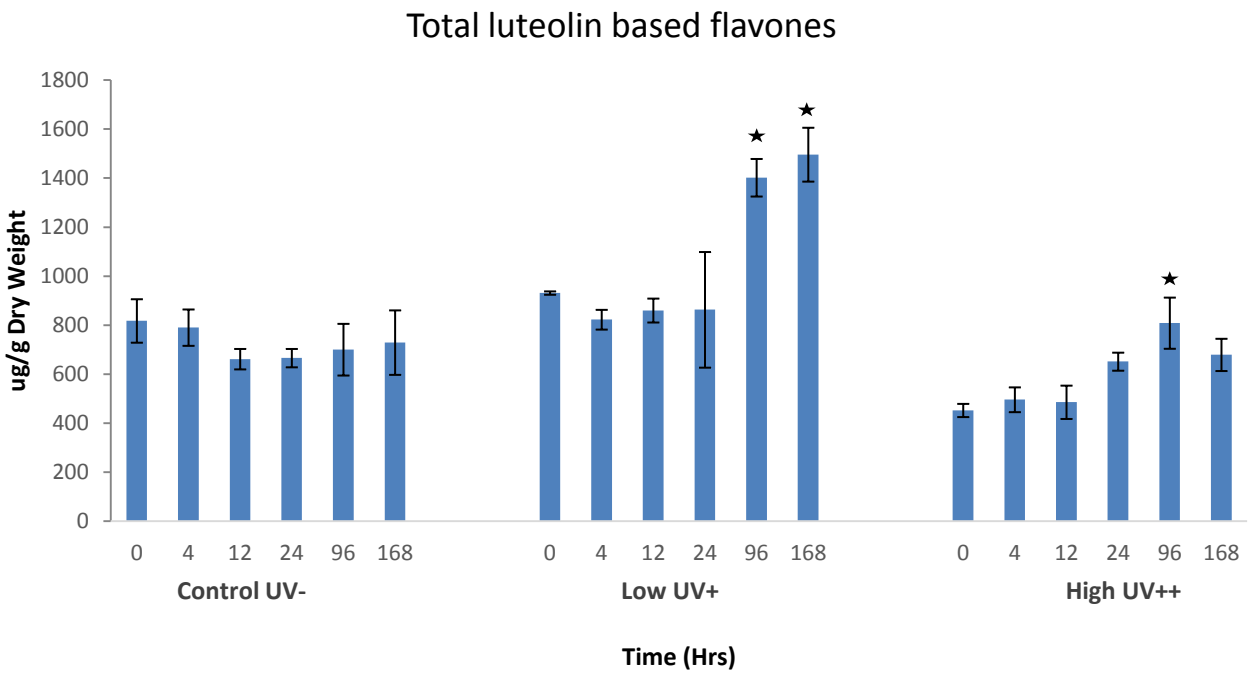



Figure 4.4: Total luteolin-based flavones produced by *M. polymorpha* under UV-B treatment. Total luteolin flavones were measured by UPLC for treated thallus tissue. (**P*<0.05, ***P*<0.01). Error bars = Standard error (n=3)

The ratio of apigenin to luteolin was determined to know if it would change under the different UV-B treatments. The ratio of Apigenin to luteolin was steady in the untreated thallus tissue at an average of around 0.75. The ratio of apigenin to luteolin was significantly higher after 24 hrs in low fluence treated tissue as compared to 0 hrs. This higher ratio was also significant at 96 and 168 hrs. High fluence treated thallus showed a significant increase in the ratio of apigenin to luteolin at 24 hrs as compared to 0 hrs (Fig. 4.5).

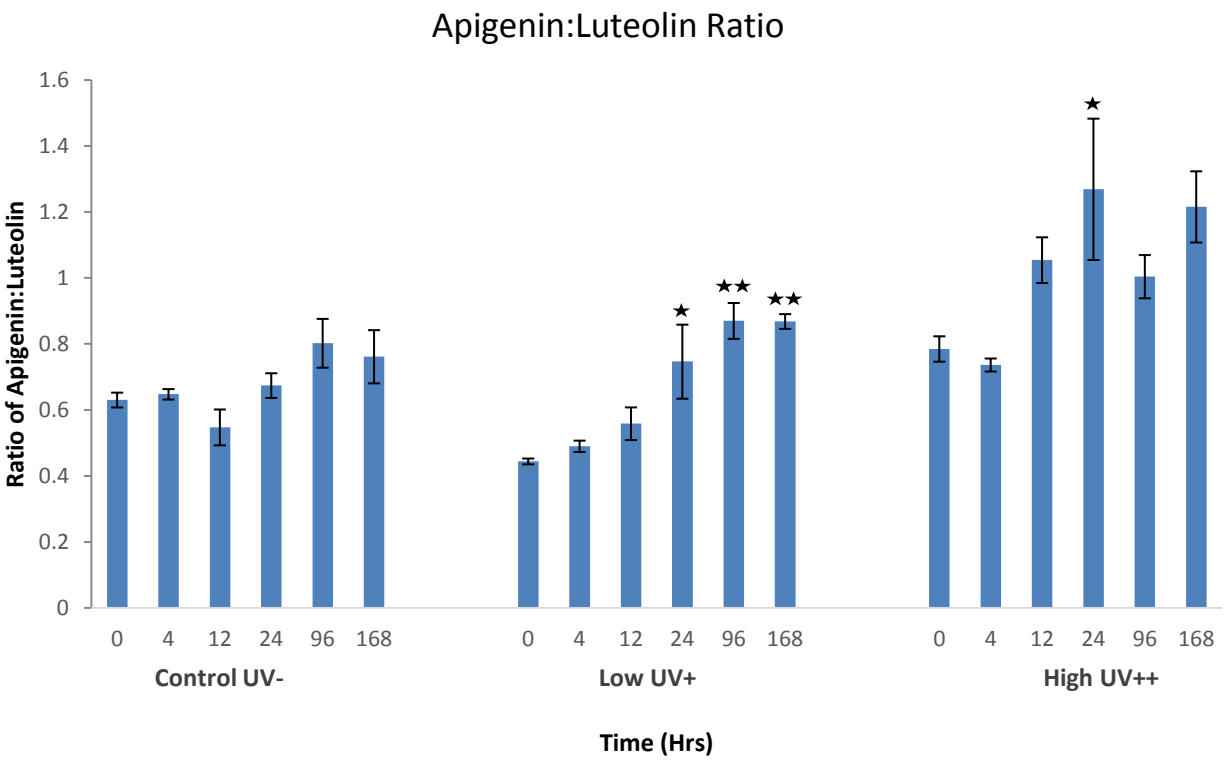



Figure 4.5: Apigenin to luteolin ratio in *M. polymorpha* under UV-B treatments. The ratio of Apigenin to luteolin was determined using UPLC and plotted over time for each treatment. (**P*<0.05, ***P*<0.01). Error bars = Standard error (n=3)

Both low and high fluence treatments showed significant increases in the total, apigenin and luteolin amounts at 96 hours, which corresponded to the peak in accumulation of these compounds. To determine the amount of change that resulted from each treatment the fold change from 0 hrs to 96 hrs was assessed for total, apigenin and luteolin flavone amounts (Fig. 4.6). Fold change of untreated tissue between 0 and 96 hours remained around 1 indicating no increases in the amounts of total, apigenin or luteolin flavones. Low fluence treated samples showed a total flavone increase of 2 fold of which the apigenin-based flavones increased 3 fold and the luteolin-based flavones increased by 1.5 fold. High fluence treated tissue showed an increase of 2 fold for total flavones, while apigenin-based flavones increased by 2.3 fold and luteolin-based flavones increased by 1.8 fold (Fig. 4.6).

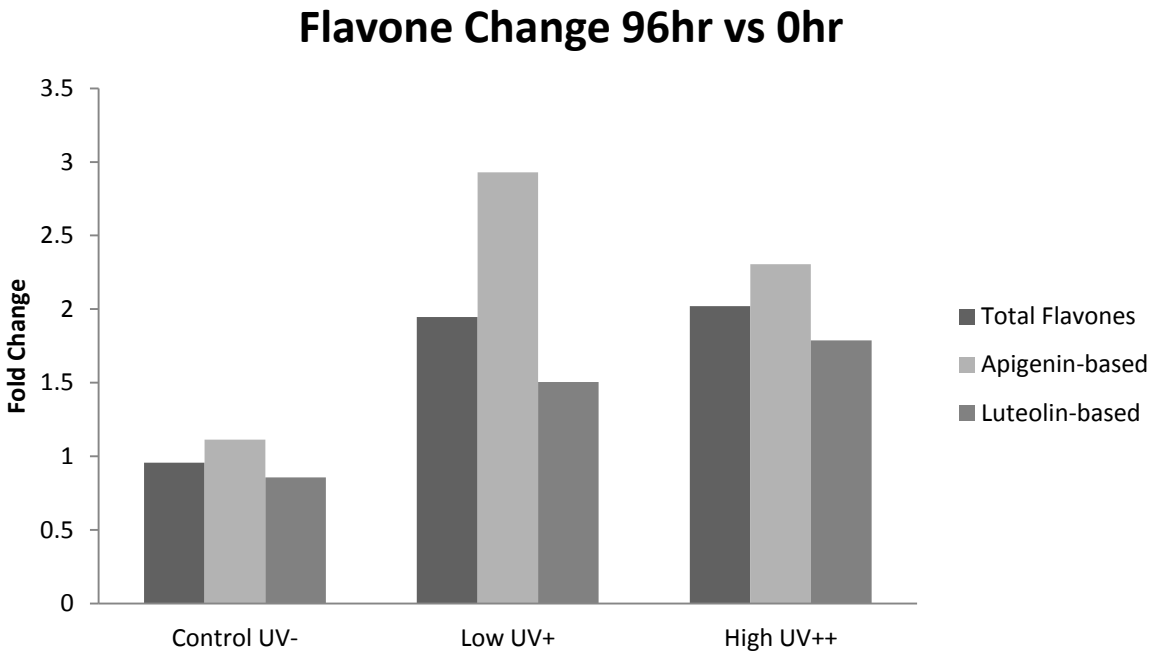



Figure 4.6: Total change of flavones between 0 hour and 96 hours of treatment. The amount of each flavone, including total flavones, was measured at 96 hours, which corresponded to the peak in production, and compared to 0 hour samples. Fold change for each treatment was plotted for 96 hr vs 0 hr.

The fold change at 96 hours between apigenin to luteolin was expressed as a ratio to determine if a significant difference ($P<0.05$) in the accumulation of each flavone occurred under the different treatments (Fig. 4.7). At 96 hours low fluence treated plants had produced 2 fold as much apigenin as compared to luteolin. In comparison high fluence plants had produced only 1.25 fold as much apigenin as compared to luteolin (Fig. 4.7).

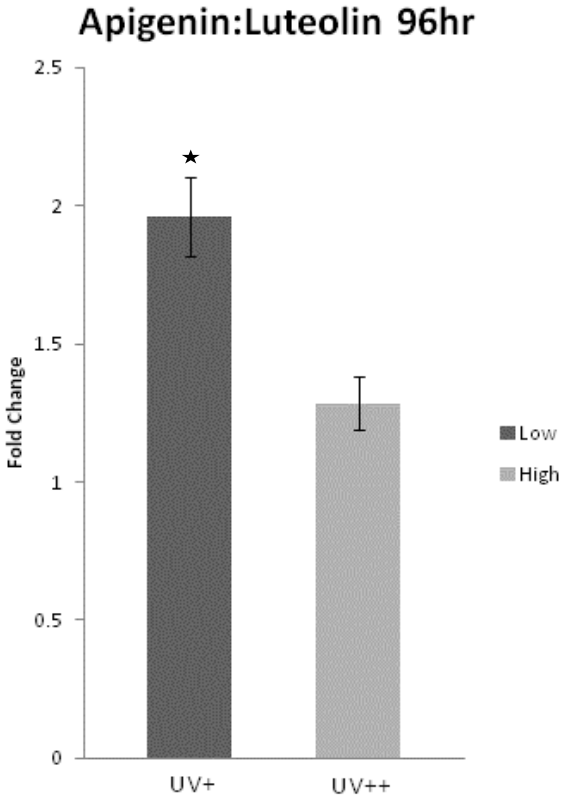



Figure 4.7: Difference ratio of fold change of apigenin to luteolin at 96 hours of *M. polymorpha* under high and low fluence UV-B. Fold change ratio of apigenin and luteolin was compared to the fold change ratio of apigenin to luteolin at 96 hours. (**P*<0.05, ***P*<0.01). Error bars = Standard error (n=3)

## 4.3 Flavonoid localisation in *M. polymorpha* under UV-B conditions

*M. polymorpha* responds to UV-B through the production of UV-B absorbing compounds including flavonoids, in particular flavones. To understand how these may provide protection the localisation of flavonoids was analysed by using a flavonoid specific stain diphenylboric acid 2-aminoethyl ester (DPBA), which fluoresces when bound to flavonoid compounds. Control, high, and low fluence treated thallus were stained and visualised by fluorescent microscopy at 96 hrs post treatment which corresponded to the highest accumulation of both total UV-B absorbing compounds and flavones specifically. It was seen that flavonoids were present on the surface of thallus tissue in the untreated plants (Fig. 4.8). Flavonoids were seen to be present in the surface scales and were particularly localised in the air pores

(Fig 4.8). The air pores of both high and low fluence treated thallus tissue continued this same trend of high accumulation in the air pores. Accumulation of flavonoids was seen in the surface scales of both high and low fluence thallus above that of the untreated control (Fig. 4.8). In high fluence treated thallus the accumulation of flavonoids in the surface was restricted to localised areas on the thallus surface while the accumulation of flavonoids in low fluence tissues was largely spread over the whole thallus (Fig. 4.8).

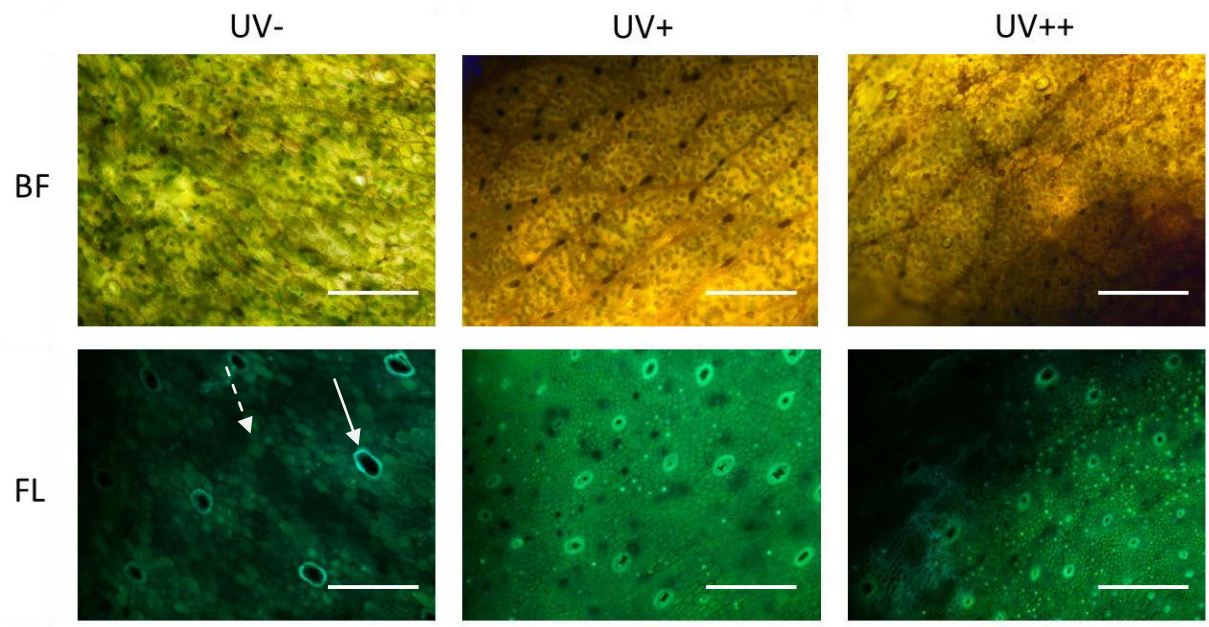



Figure 4.8: Visualisation of flavonoid accumulation under UV-B treatments in *M. polymorpha*. Thallus tissue was stained with diphenylboric acid 2-aminoethyl ester (DPBA) which binds flavonoids and can be seen by green fluorescence. Both fluorescent (FL) and bright field (BF) images of thallus treated with no UV-B control (UV-), high fluence (UV++) and low fluence (UV+) are shown. The air pores of thallus tissue are enriched for flavonoids (solid arrow), while flavonoids also locate to the surface scales (dashed arrow) Images were taken after 96 hrs of the start of treatment. Fluorescent image exposures 300ms. Scale bar = 200um

Low fluence plants showed the highest increases in the accumulation of flavonoids and the localisation in the thallus was further analysed (Fig. 4.9). The accumulation of flavonoids was largely restricted to the scales of the thallus and air pores. This covered large areas of the thallus surface. Transverse sections showed that the flavonoids were localised largely

around the mesophyll cells and accumulated highly in the epidermal layers of the thallus (Fig. 4.9).

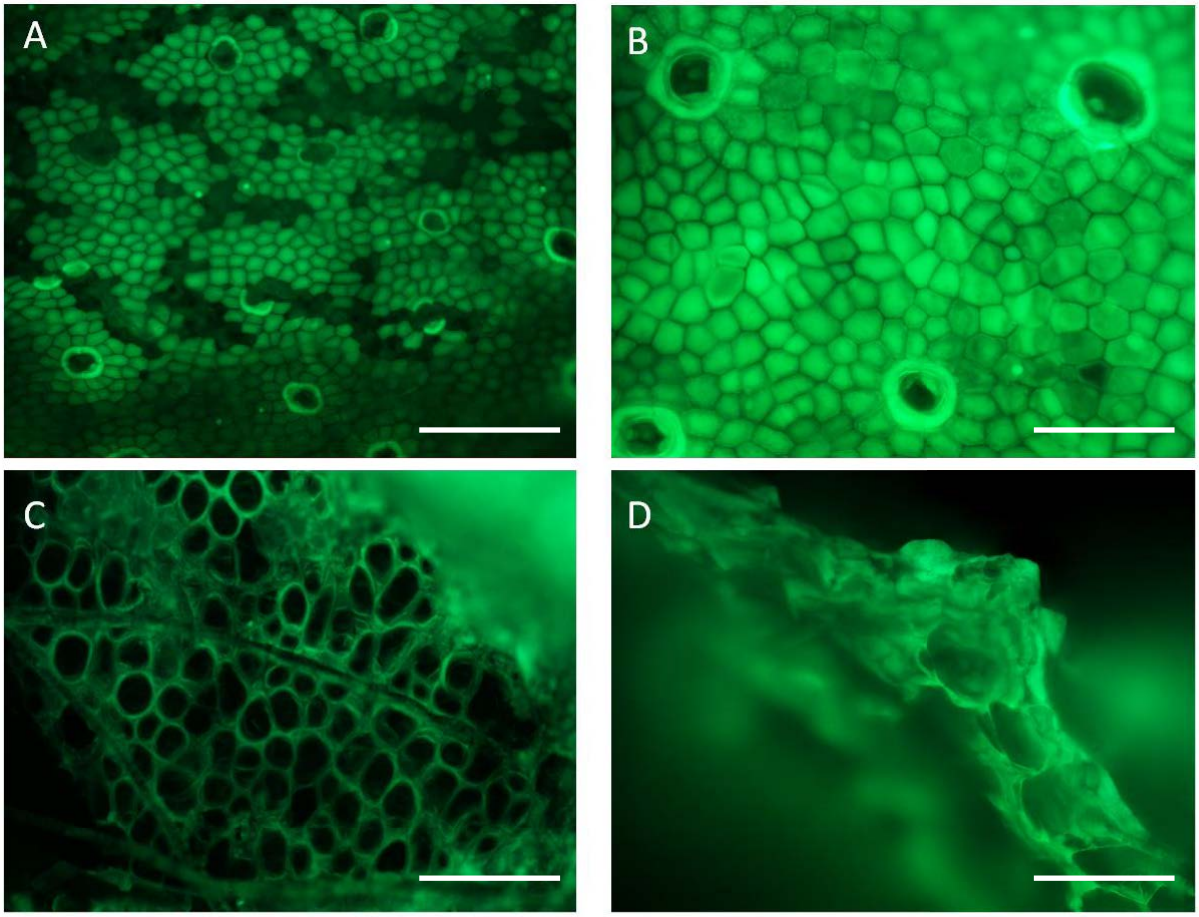



Figure 4.9: Fluorescent micrographs of *M. polymorpha* thallus after 96 hours of starting low fluence UV-B treatment (UV+), stained with flavonoid specific DPBA which can be seen by green fluorescence. A,B) Thallus surface showing high levels of fluorescence indicating accumulation of flavonoids in surface layers, particularly the scales. C) Cross Section of thallus tissue showing flavonoid accumulation in areas between cells. D) Cross section of thallus showing accumulation of flavonoids around cells and within epidermal layers. Image exposures 300ms. Scale bar = (A) 200 µm, (B,C,D) 100 µm.

Gemmae are the main reproductive tissue of *M. polymorpha* and therefore may be important to protect against damaging UV-B. Gemmae were stained and analysed to determine the flavonoid localisation in these structures after UV-B treatment (Fig. 4.10). Untreated gemmae showed little accumulation of flavonoids within the gemmae tissue as a

whole but oil cells within the gemmae fluoresced brightly. High and low fluence treated gemmae showed accumulation of flavonoids within the tissues as well as the previously seen fluorescence of oil cells. The intensity of gemmae fluorescence was equal between both high and low fluence treated gemmae structures which may indicate amounts of flavonoids are similar between both treatments (Fig. 4.10)

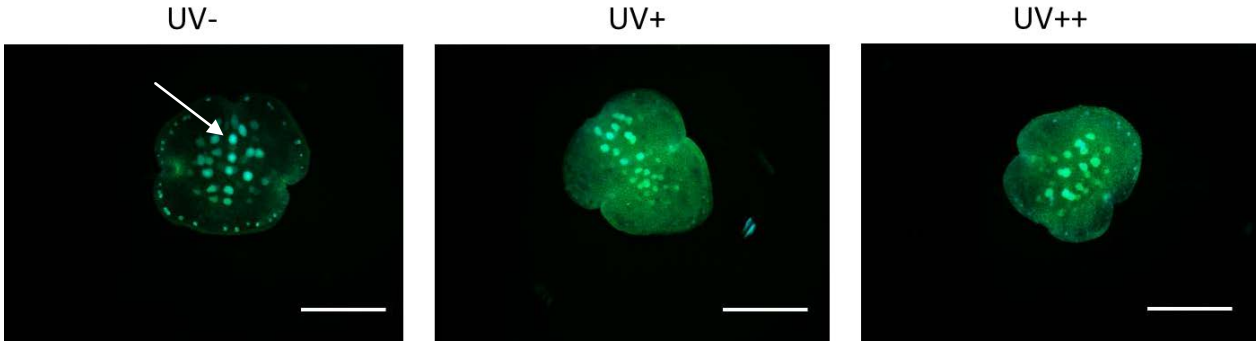



Figure 4.10: Flavonoid accumulation in gemmae. Fluorescent micrographs of *M. polymorpha* gemmae after 96 hours of starting UV-B treatment, stained with flavonoid specific DPBA which can be seen by green fluorescence. Oil cells within gemmae fluoresce brightly (arrow). Scale bar = 200um

Flavonoids seen in the reproductive tissues may indicate a protective function for new tissues so the thallus apical notch, where new thallus growth occurs, was also stained and visualised for flavonoid accumulation. Accumulation could be seen in all treatments including that of non-UV-treated thallus tissue. However flavonoids were only enriched in untreated thallus around the apical notch and were not seen in older thallus tissue (Fig. 4.11). This pattern was also seen in high fluence treated thallus with an increased accumulation of flavonoids around the apical notch while flavonoid fluorescence was largely absent from older tissues. Low fluence treatments also showed high accumulation in the apical notch tissue yet flavonoid fluorescence was also seen in the surface cells of the older tissues as well (Fig. 4.11).

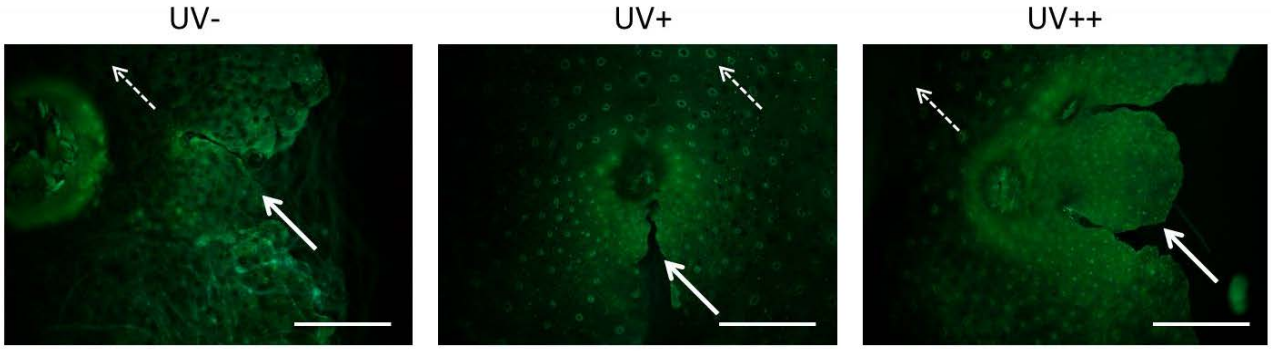



Figure 4.11: Flavonoid accumulation in growing thallus. Fluorescent micrographs of *M. polymorpha* apical notch regions after 96 hours of starting UV-B treatment, stained with flavonoid specific DPBA which can be seen by green fluorescence. White solid arrow indicates area of newly growing thallus tissue at the apical notch. Dashed arrows indicate older thallus tissue. Scale bar = 500um

## 4.4 Discussion

We found that under UV-B treatment *M. polymorpha* produced UV-B absorbing compounds and flavone compounds in response to UV-B. *M. polymorpha* produced these compounds under both high and low fluence with the greatest accumulations occurring under the low fluence treatment (Fig. 4.2). Total UV-B absorbing compounds under the high fluence showed a non-significant difference to control yet the total flavones measured were significantly different (Fig. 4.1, 4.2). It cannot be ruled out that other compounds, which are not flavonoids, which absorb in the UV-B range are also produced under UV-B low fluence and may not be produced under high fluence treatment. Interestingly changes in the amount of UV-B absorbing compounds under the high fluence treatment did increase at 24 and 96 hours but not to a statistically significant level in our experiment. In a previous study of *M. polymorpha* under increasing UV-B it was found no increase in flavonoid levels occurred with increasing UV-B (Markham et al., 1998a). It was found, however, that there was a strong correlation between increasing UV-B and an increase in the ratio of luteolin to apigenin glycosides (Markham et al., 1998a). In our study we saw the ratio of apigenin to luteolin increase significantly under low fluence conditions after 24 hours of treatment yet the high fluence treatment showed only one significantly different time point at 24 hours after treatment. This differs from the previous study in that under our low fluence treatment conditions apigenin-based flavones were found to increase to higher levels rather than

luteolin-based flavones. The previous study was conducted for a much longer time and plants were grown under ambient daylight conditions in a greenhouse. The methodology used for enhancement of UV-B would have exposed plants to varying degrees of UV-B and PAR level over the course of the study and over each day as the sun rose, set or was obscured by cloud cover (Markham et al., 1998a). When grown under ambient light the high PAR levels may also induce flavonoids in such a way that increased UV-B does not induce additional production of flavonoids. Such variations may have affected the plants response and although it may reflect a natural production of flavonoids to ambient light and UV-B, the ability of plants to produce flavonoids in response to a direct and sustained UV-B enhancement would have been hard to observe. In our study we use controlled environmental conditions that do not vary and allow us to measure the response to known and constant UV-B levels and therefore may allow us to better understand the ability of *M. polymorpha* to produce flavonoid compounds in direct response to UV-B.

The ratio of apigenin to luteolin in our study showed that an increase in apigenin may be important for the low fluence acclimatisation over multiple days (Fig. 4.5). High fluence treatment showed an increase only at 24 hours and interestingly this is directly after the UV-B irradiance of high fluence plants. It may be that UV-B sensing favours the production of apigenin-based flavones and its accumulation seen at these time point coincides with this. The luteolin-based flavones increased in both low and high fluence treatments and were at the highest at the 96 hour peak accumulation time point. Taking the fold change at this time point we can see that the fold change accumulation between high and low fluence treatments for total flavones is very similar yet the fold change contribution from apigenin and luteolin is very different (Fig. 4.6). The fold change of apigenin under low treatment is very high while under high UV-B treatment we see that luteolin contributes more and apigenin less (Fig. 4.6). Directly comparing this difference at the 96 hour time point we see a statistically significant difference in the fold change ratio for apigenin to luteolin production between the low and high UV-B treatments (Fig. 4.7). This shows that apigenin is produced at a lower rate and luteolin at a higher rate in high fluence UV-B treatment as compared to the low fluence UV-B treatment at 96 hours, which favours apigenin production. The mechanism to produce more apigenin or luteolin under varying UV-B is difficult to determine as they may both be produced through the UVR8 pathway initially under UV-B irradiance but

once UV-B light is removed in the high fluence treatment the mechanisms that continue to produce flavonoids or convert flavones between apigenin or luteolin may differ to that of low fluence plants that, still under UV-B irradiance, may continue to use the direct UVR8 pathway for flavonoid production. The conversion of apigenin to luteolin could be facilitated through flavonoid 3-hydroxylase (F3H) (Schwinn *et al.*, 2014). Whether *M. polymorpha* has an F3H enzyme capable of this function is unknown. Future work to determine the effect of UV-B and ROS on plants that may lack conversion between apigenin and luteolin, potentially through mutation of candidate F3H genes could be particularly important.

This difference in apigenin to luteolin was found previously and it was suggested that the increase in luteolin was due to a higher need for ROS scavenging in UV-B treated plants (Markham et al., 1998a). It has been found that B-ring ortho-dihydroxyflavones like luteolin are known to be significantly more effective free radical scavengers/ antioxidants than B-ring mono-hydroxyflavones such as apigenin (Montesinos *et al.*, 1995, Heim et al., 2002). We found that luteolin may be more important in high UV-B treated plants rather than low UV-B treated plants and also that ROS species in high fluence treatments are seen to increase while low UV-B treatments showed no increases in ROS as compared to the UV-B lacking control (Chapter 3). It may be that ROS are scavenged in low fluence treated plants by flavones that are already present such as luteolin or apigenin and thus we fail to see any detectable increase in ROS. Alternatively our detection method may not be precise enough to measure any slight changes in the ROS level of low fluence plants. However in the high fluence treatment we see a very distinct rise in ROS and this increase may be observed as ROS are produced at a rate higher than the ROS scavengers such as luteolin can deal with them. The damage caused by the initial high fluence UV-B treatment may continue past day 1 and thus an increased need for more effective ROS scavengers such as luteolin rather than apigenin may result in the increased accumulation of luteolin seen in high fluence plants as compared to low fluence which may not have the same damage or corresponding ROS burden.

Flavonoids that are induced by UV-B may have functions to reduce the effects of UV-B damage which include both ROS scavenging and also UV-B screening properties. In particular the flavones absorb wavelengths within the UV-B range of 280-320 nm which makes them

ideal for the use in UV-B screening. Using a fluorescent reporter we were able to view the localisation of flavonoids that were produced under the different UV-B treatments and found a large amount were located in the epidermal layers which indicates a large proportion of these flavonoids may act in UV-B screening. This was especially apparent in the low fluence plants which had high levels of UV-B induced production of flavones (Fig. 4.9). This production occurred largely in the epidermal layers and was also seen to form around cells in cross sections of the thallus tissue (Fig. 4.9).

In low fluence plants we see apigenin is increased and we also see flavonoids localised in the surface layers. Apigenin has a higher molecular extinction coefficient over wavelengths of 290-320 nm than luteolin (Agati *et al.*, 2013). While apigenin and luteolin both absorb at similar wavelengths in the UV range this increase in the extinction coefficient of apigenin may make it a much better UV-B screening compound. This could help explain high levels of apigenin-based flavone production in low fluence plants as compared to high fluence plants. Low fluence may induce greater levels of apigenin flavones which are used predominately for UV-B screening, removing the potential for UV-B to damage underlying tissues and induce ROS. Whereas high fluence plants cannot initially deal with the high level of UV-B so damage may occur immediately by overwhelming innate screening ability without giving plants time to increase any UV-B screening potential. ROS are subsequently highly induced and so the need for UV-B screening compounds such as apigenin are produced less in favour of ROS scavenging flavones such as luteolin in order to deal with this ROS burden. The contribution of flavonoids and in particular luteolin-based flavones that have higher ROS scavenging function have been found to be important in high light and UV-B treatments (Agati et al., 2011, Agati et al., 2009). Unfortunately the fluorescent marker used in our study is unable to differentiate between apigenin or luteolin-based flavones so we cannot determine whether apigenin-based flavones are used in epidermal layers and luteolin flavones in the deeper tissues, which could help provide evidence for each flavones' function. The accumulation of flavonoids in surface layers of high fluence plants was seen to be lower than in that of low fluence plants (Fig. 4.8, 4.11), which further indicates a lower contribution of UV-B screening in high fluence treatments as compared to low fluence. Flavonoids were not seen within the vacuoles of thallus tissue but this may have been due to sampling of the thallus tissue releasing vacuole contents in cross section analysis. The high accumulation of flavonoids in surface layers may have also prevented visualisation of

localisation in deeper tissues. Confocal analysis of flavonoid localisation would help to show the localisation in deeper tissues and this would be especially important in high fluence treated plants due to the previously seen visualisation of ROS production in the chloroplasts. Chloroplast localised flavonoids may also play an important role in the reduction of ROS by scavenging singlet oxygen production (Agati *et al.*, 2007).

Flavonoid localisation was also seen in the apical notch of thallus tissue and also the reproductive gemmae (Fig. 4.10, 4.11). This represents the youngest tissues of the plant and localisation to these areas may be important under UV-B stress. In particular the increased flavonoid accumulation in gemmae under both high and low fluence treatments as compared to the UV-B lacking control may be important for the protection of the reproductive structures. Gemmae from treatments that showed accumulation were plated directly under light that included UV-B alongside those from the UV-B lacking control which did not show accumulation, yet no difference in growth was observed (data not shown). This may indicate that accumulation of flavonoids in gemmae may help protect them whilst forming in plants but the priming of flavonoid accumulation in gemmae does not give them an advantage in UV-B enhanced environments. Once plated gemmae that have not been primed with flavonoid accumulation may rapidly respond to produce their own protective flavonoid compounds for protection and thus no difference in growth over time is observed. Similarly the localisation of flavonoids compounds in the apical notch may help to protect the new tissue from damage by UV-B. Auxin regulation is another function of flavonoids and may also play an important role in these reproductive tissues. Flavonoids have been found to be positive regulators of auxin transport inhibitors and allow for the accumulation of auxin in specific tissues (Peer & Murphy, 2007, Peer *et al.*, 2001). While they may play an important role in development, the determinant function of accumulations of flavonoids as either auxin regulators, UV-B screeners or ROS scavengers in plant tissues is hard to determine. In our study the flavonoid accumulation we see in the apical notch and gemmae tissue in response to UV-B may indeed function in helping regulate auxin and help development in these younger tissue under stress. However it is much more likely these accumulations have UV-B screening or ROS scavenging activity, especially given the large changes in flavonoid levels we observe.

Overall we observe that *M. polymorpha* responds to UV-B through the production of UV-B absorbing and flavones compounds. These compounds may largely contribute to UV-B

screening in low fluence treatments and a role for ROS scavenging may also be important, especially in high fluence treated plants. This increase in UV-B screening may have been an important mechanism to deal with incident UV-B radiation of early land plants, of which *M. polymorpha* is the closest extant relative.

# Chapter 5: The genetic response of *M. polymorpha* to UV-B

## 5.1 Introduction

*M. polymorpha* exposure to UV-B at both high and low fluence resulted in the accumulation of UV-B absorbing compounds including flavonoids, in particular flavones. This accumulation would likely result from the activation of a flavonoid pathway mediated through the UVR8 photoreceptor which is responsible for the UV-B response in higher plants (Jenkins, 2014). While UVR8 is the photoreceptor required for UV-B signalling, a suite of other factors may be required for the full acclimation response. In particular the UV-B response is known to be regulated by a number of key factors such as repressor of UV-B photomorphogenesis (RUP1), elongated hypocotyl 5 (HY5) and constitutively photomorphogenic 1 (COP1) (Favory et al., 2009, Brown & Jenkins, 2008). The key biosynthetic genes required for the production of flavones include phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), chalcone synthase (CHS), chalcone isomerise (CHI) and flavones synthase (FNS). While the key genes related to the direct UV-B response may respond directly to UV-B, a number of other genes may also be required to mitigate the indirect effects of UV-B such as the production of ROS or DNA damage.

To determine whole plant changes in gene expression, *M. polymorpha* was subject to high fluence and sampled at 4 hours and 1 day. The 4 hour time point was used to determine early UV-B responses involved the specific UV-B pathway while 1 day was used to determine later responses that may involve the non-specific pathway. Low fluence plants were sampled at 1 day also. High fluence 4 hour samples were under direct UV-B irradiance while 1 day samples had undergone a 12 hour treatment of UV-B followed by a dark period and where used to determine the stress response to each level of UV-B fluence. All samples were then RNA sequenced to determine the changes in transcript abundance that occur under the different UV-B treatments and the corresponding gene regulation that results in the UV-B response in *M. polymorpha*.

## 5.2 Results

### 5.2.1 RNA sequencing of *M. polymorpha* under UV-B conditions

RNA sequencing of high fluence plants at the time point of 4 hours, early in the UV-B response and under direct UV-B irradiation revealed a number of up-regulated genes (Table 5.1). The most highly up-regulated gene was a putative copper chaperone (Mapoly0025s0089.1) and this was also up-regulated at the 1 day time point in both high and low fluence treatments (Table 5.2, 5.3). Under UV-B, differentially expressed genes were found that corresponded to the known regulators of the UV-B response such as RUP1 (Mapoly0094s0072.1), and HY5 (Mapoly0001s0021.1) and chalcone synthase (Mapoly0021s0159.1). A putative NAD dependent epimerase/dehydratase family gene (Mapoly0190s0002.1) was found to be up regulated at 4 hours and 1 day of high UV-B. Other putative genes that were up-regulated at 4 hrs of UV-B included a DNA glycosylase (Mapoly0074s0054.1) and multiple chlorophyll A-B binding proteins (Table 5.1). The most down-regulated gene coded a putative chlorophyllase protein (Mapoly0008s0178.1). A putative chalcone/stilbene synthase was also found to be in the bottom 20 down regulated genes (Mapoly0041s0118.1).

Table 5.1: The 20 most up regulated and down regulated genes in *M. polymorpha* under high fluence UV-B treatment at 4 hours. ** indicates genes shared across all time data sets. *H indicates gene shared in only high fluence data sets. Mpoly_primaryTs is the unique identifier. padj is the adjusted p value. Base mean shows the mean base reads across each data set for a given gene model.

| | Mpoly_primaryTs | log2Fold Change | padj | base mean | Possible gene function |
|---|---|---|---|---|---|
| ** | Mapoly0025s0089.1 | 4.39 | 1.61E-114 | 446 | Copper chaperone |
| | Mapoly0034s0071.1 | 4.11 | 5.32E-289 | 875 | Fatty acid desaturase, Chloroplast-like |
| | Mapoly0094s0072.1 | 3.84 | 0 | 1788 | WD40 repeat protein (RUP1) |
| | Mapoly0047s0106.1 | 3.81 | 0 | 3564 | Chlorophyl A-B binding protein |
| | Mapoly0074s0054.1 | 3.58 | 2.53E-143 | 296 | DNA glycosylase |
| | Mapoly0041s0144.1 | 3.39 | 6.91E-18 | 21 | Chlorophyl A-B binding protein |
| | Mapoly0001s0021.1 | 3.38 | 8.80E-293 | 908 | bZip transcription factor (HY5) |
| *H | Mapoly0190s0002.1 | 3.14 | 1.59E-127 | 647 | NAD dependent epimerase/dehydratase family |
| | Mapoly0081s0077.1 | 3.12 | 9.94E-268 | 828 | pyridoxal 5'-phosphate synthase |
| | Mapoly0035s0135.1 | 3.12 | 4.57E-64 | 123 | EF-hand domain pair |
| | Mapoly0019s0086.1 | 3.02 | 4.45E-234 | 700 | Tetratricopeptide repeat (TPR)-like superfamily protein |
| | Mapoly0021s0159.1 | 3.01 | 6.47E-141 | 5134 | Chalcone synthase |
| | Mapoly0049s0045.1 | 2.93 | 4.88E-102 | 339 | Unknown |
| | Mapoly0012s0089.1 | 2.93 | 6.34E-13 | 8 | tRNA-isopentenyltransferase (IPT5) |
| | Mapoly0021s0110.1 | 2.89 | 2.67E-14 | 19 | Ankyrin repeat and protein kinase domain containing protein |
| | Mapoly0041s0138.1 | 2.87 | 9.02E-13 | 11 | Chlorophyl A-B binding protein |
| | Mapoly0007s0046.1 | 2.86 | 1.54E-84 | 271 | Chlorophyll A-B binding protein |
| | Mapoly0016s0142.1 | 2.78 | 8.98E-176 | 476 | ABC transport 1 family |
| | Mapoly0007s0213.1 | 2.77 | 0 | 1881 | Sigma factor |
| | Mapoly0067s0074.1 | 2.73 | 6.52E-34 | 75 | Predicted small molecule transporter |
| | Mapoly0049s0030.1 | 2.69 | 3.20E-49 | 144 | B-box zinc finger protein CONSTANS like |
| | | | | | |
| | Mapoly0008s0178.1 | -2.40 | 1.31E-20 | 49 | Chlorophllyase protein |
| | Mapoly0010s0221.1 | -2.18 | 8.66E-16 | 143 | GDSL-like Lipase/Acylhydrolase |
| | Mapoly0083s0098.1 | -2.10 | 1.59E-38 | 169 | Leucine rich repeat receptor kinase |
| | Mapoly0118s0002.1 | -2.09 | 5.50E-29 | 127 | Leucine rich repeat receptor kinase |
| | Mapoly0010s0216.1 | -2.04 | 1.39E-09 | 199 | Unknown |
| | Mapoly0050s0045.1 | -1.96 | 1.63E-15 | 203 | Plasma membrane ATPase |
| | Mapoly0098s0055.1 | -1.94 | 4.53E-06 | 7 | Unknown |
| | Mapoly0042s0125.1 | -1.92 | 1.70E-06 | 17 | Esterase/lipase |
| | Mapoly0126s0004.1 | -1.92 | 4.98E-08 | 327 | Sodium/phosphate symporter |
| | Mapoly0045s0001.1 | -1.91 | 2.47E-08 | 60 | Germin like protein 1 |
| | Mapoly0033s0171.1 | -1.90 | 8.42E-11 | 60 | Unknown |
| | Mapoly0225s0001.1 | -1.89 | 8.04E-13 | 830 | Unknown |
| | Mapoly0007s0179.1 | -1.84 | 6.46E-09 | 264 | Wall associated receptor kinase |
| | Mapoly0007s0136.1 | -1.84 | 2.79E-15 | 200 | tRNA ligase |
| | Mapoly0126s0023.1 | -1.82 | 1.48E-07 | 58 | Sodium/phosphate symporter |
| | Mapoly0097s0010.1 | -1.80 | 2.91E-07 | 69 | Pathogenisis-related protein |
| | Mapoly0007s0207.1 | -1.77 | 2.21E-08 | 33 | Unknown |
| | Mapoly0057s0107.1 | -1.75 | 4.72E-132 | 2286 | Unknown |
| | Mapoly0041s0118.1 | -1.71 | 2.84E-08 | 619 | Chalcone/stilbene synthase |
| | Mapoly0243s0005.1 | -1.71 | 6.80E-06 | 28 | Peroxidase |
| | Mapoly0196s0008.1 | -1.70 | 1.63E-13 | 201 | Hevamine-A like |

High fluence treated plants were also sampled at 1 day and RNA sequenced. This represented the whole plant response to UV-B treatment and the mitigation of any stress as plants were not directly under UV-B irradiation at this time point. The highest up-regulated gene was that of a heat shock protein (Mapoly0076s006.1), which was not shared with either the low fluence 1 day or high fluence 4 hour data sets (Table 5.2). The most down-regulated gene was that of a putative carbonic anhydrase (Mapoly0012s0071.1). The copper chaperone (Mapoly0025s0089.1) that is shared in all data sets was also highly up-regulated. A number of genes were also shared with the low fluence 1 day data set (Table 5.2, 5.3). This included a signal transduction adapter molecule (Mapoly0039s0049.1), NA P-type ATPase (Mapoly0106s0039.1), EamA-like transporter (Mapoly0003s0109.1), tRNA-splicing ligase RtcB (Mapoly0034s0109.1), unknown protein (Mapoly0041s0017.1) and a multicopper oxidase (Mapoly008s0270.1). An ethylene responsive transcription factor (ERF) (Mapoly0166s0010.1) was also in the top three most up-regulated genes in the high fluence 1 day data set.

Low fluence treatment at 1 day which represented the whole plant response to UV-B, shared many different genes that were conserved in the high fluence response (Table 5.3). The highest up-regulated gene was a putative copper chaperone (Mapoly0180s0021.1) and the most down-regulated was that of a putative peroxidase (Mapoly0157s0026.1). A large number of peroxidase genes were found to be both highly up and down-regulated in the low fluence 1 day data set. Multiple putative copper chaperone genes were also found to be highly up-regulated in the low fluence data set (Table 5.3).

Table 5.2: The 20 most up regulated and down regulated genes in *M. polymorpha* under high fluence UV-B treatment at 1 day. * indicates genes common between high and low fluence at 1 day. ** indicates genes shared across all data sets. *H indicates gene shared in only high fluence data sets. Mpoly_primaryTs is the unique identifier. padj is the adjusted p value. Base mean shows the mean base reads across each data set for a given gene model.

| | Mpoly_primaryTs | log2Fold Change | padj | base mean | Possible gene function |
|---|---|---|---|---|---|
| | Mapoly0076s0006.1 | 6.20 | 1.12E-122 | 183 | Heat shock protein |
| | Mapoly0140s0002.1 | 6.06 | 1.29E-234 | 587 | Unknown |
| | Mapoly0166s0010.1 | 5.66 | 3.45E-108 | 160 | Ethylene responsive transcription factor |
| *H | Mapoly0190s0002.1 | 5.29 | 2.72E-235 | 647 | NAD dependent epimerase/dehydratase family |
| ** | Mapoly0025s0089.1 | 5.25 | 1.98E-210 | 446 | Copper chaperone |
| | Mapoly0044s0085.1 | 4.69 | 3.90E-73 | 98 | WRKY Interacting |
| * | Mapoly0039s0049.1 | 4.69 | NA | 3214 | Signal transduction adapter molecule |
| | Mapoly0006s0218.1 | 4.68 | 1.78E-49 | 51 | Glutamine repeat protein |
| * | Mapoly0106s0039.1 | 4.65 | 2.14E-221 | 600 | Na P-type ATPase |
| * | Mapoly0003s0267.1 | 4.64 | 9.23E-40 | 302 | EamA-like transporter family |
| * | Mapoly0034s0109.1 | 4.53 | 4.45E-143 | 248 | tRNA-splicing ligase RtcB |
| * | Mapoly0041s0017.1 | 4.52 | 6.93E-61 | 77 | Unknown |
| | Mapoly1326s0001.1 | 4.47 | 3.17E-72 | 149 | Unknown |
| | Mapoly0052s0127.1 | 4.35 | 7.00E-50 | 65 | Leucine rich repeat protein kinase |
| | Mapoly0001s0098.1 | 4.15 | 6.27E-134 | 237 | L-type lectin domain containing receptor kinase |
| | Mapoly0180s0030.1 | 3.97 | 1.59E-91 | 378 | Germin-like protein |
| | Mapoly0004s0080.1 | 3.61 | 4.91E-38 | 63 | Pectinesterase |
| | Mapoly0045s0146.1 | 3.60 | 2.09E-117 | 295 | Heat shock protein mitochondrial-like |
| * | Mapoly0008s0270.1 | 3.60 | 2.01E-44 | 111 | Multicopper oxidase |
| | Mapoly0021s0041.1 | 3.57 | 4.05E-59 | 83 | Polyphenol oxidase |
| | Mapoly0097s0053.1 | 3.49 | 5.34E-20 | 17 | ABC transporter G family |
| | | | | | |
| | Mapoly0012s0071.1 | -3.13 | 2.55E-100 | 299 | Carbonic anhydrase |
| | Mapoly0030s0112.1 | -2.65 | 1.37E-50 | 183 | Sodium/calcium exchanger protein |
| | Mapoly0131s0030.1 | -2.49 | 2.49E-101 | 385 | Nitrate transporter |
| | Mapoly0054s0123.1 | -2.42 | 1.73E-13 | 26 | Sodium/phosphate symporter |
| | Mapoly0121s0010.1 | -2.24 | 1.04E-43 | 488 | Unknown |
| | Mapoly0004s0019.1 | -2.08 | 2.84E-29 | 139 | Unknown |
| | Mapoly0083s0032.1 | -2.05 | 2.98E-11 | 558 | Unknown |
| | Mapoly0077s0034.1 | -2.03 | 8.37E-66 | 311 | Esterase/lipase |
| | Mapoly0052s0090.1 | -1.92 | 1.06E-21 | 109 | Oxidoreductase |
| | Mapoly0027s0164.1 | -1.92 | 2.43E-09 | 78 | Unknown |
| | Mapoly0007s0096.1 | -1.89 | 7.27E-119 | 1942 | Pectate lyase |
| | Mapoly0137s0006.1 | -1.89 | 1.79E-123 | 2118 | Unknown |
| | Mapoly0044s0057.1 | -1.87 | 1.73E-72 | 606 | Iron permease |
| | Mapoly0001s0357.1 | -1.86 | 1.40E-119 | 787 | Sulfate transporter |
| | Mapoly0126s0024.1 | -1.84 | 3.90E-10 | 819 | Phosphate transporter |
| | Mapoly0033s0103.1 | -1.81 | 3.63E-17 | 90 | Extensin |
| | Mapoly0047s0055.1 | -1.80 | 5.47E-08 | 27 | Ferric chelate reductase |
| | Mapoly0121s0008.1 | -1.80 | 2.15E-16 | 912 | Unknown |
| | Mapoly0022s0134.1 | -1.79 | 3.40E-41 | 330 | Expansin |
| | Mapoly0129s0043.1 | -1.74 | 1.55E-06 | 20 | Cytochrome P450 |
| | Mapoly0211s0009.1 | -1.72 | 1.09E-05 | 14 | Unknown |

Table 5.3: The 20 most up regulated and down regulated genes in *M. polymorpha* under low fluence UV-B treatment at 1 day. * indicates genes common between high and low fluence at 1 day. ** indicates genes shared across all data sets. Mpoly_primaryTs is the unique identifier. padj is the adjusted p value. Base mean shows the mean base reads across each data set for a given gene model.

| | Mpoly_primaryTs | log2Fold Change | padj | base mean | Possible gene function |
|---|---|---|---|---|---|
| | Mapoly0180s0021.1 | 4.87 | 1.57E-151 | 662 | Copper chaperone |
| | Mapoly0180s0018.1 | 4.46 | 5.34E-132 | 542 | Copper chaperone |
| | Mapoly0106s0052.1 | 4.37 | 2.09E-36 | 67 | Peroxidase |
| * | Mapoly0039s0049.1 | 4.35 | 0 | 8856 | Signal transduction adapter molecule |
| | Mapoly0180s0014.1 | 4.19 | 3.49E-42 | 1453 | Copper chaperone |
| | Mapoly0006s0129.1 | 4.13 | 8.16E-97 | 726 | Unknown |
| * | Mapoly0041s0017.1 | 4.05 | 3.25E-69 | 361 | Unknown |
| ** | Mapoly0025s0089.1 | 4.04 | 2.44E-169 | 2047 | Copper chaperone |
| * | Mapoly0003s0267.1 | 4.04 | 3.81E-64 | 705 | EamA-like transporter family |
| | Mapoly0220s0001.1 | 3.99 | 1.86E-21 | 37 | Peroxidase |
| | Mapoly3855s0001.1 | 3.97 | 1E-24 | 40 | Peroxidase |
| | Mapoly0161s0003.1 | 3.95 | 2.89E-97 | 3008 | Unknown |
| | Mapoly0016s0058.1 | 3.91 | 7.25E-29 | 66 | Ile-tRNA |
| | Mapoly0008s0258.1 | 3.90 | 9.53E-32 | 905 | Potato inhibitor I family |
| * | Mapoly0034s0109.1 | 3.88 | 1.09E-141 | 818 | tRNA-splicing ligase RtcB |
| | Mapoly0106s0051.1 | 3.87 | 1.74E-25 | 53 | Peroxidase |
| | Mapoly0052s0050.1 | 3.87 | 5.42E-76 | 543 | Unknown |
| * | Mapoly0008s0270.1 | 3.77 | 1.65E-50 | 358 | Multicopper oxidase |
| * | Mapoly0106s0039.1 | 3.75 | 1.18E-179 | 1220 | Na P-type ATPase |
| | Mapoly0015s0156.1 | 3.73 | 3.37E-53 | 579 | Peroxidase |
| | Mapoly0076s0004.1 | 3.60 | 1.84E-42 | 517 | Small heat shock protein |
| | | | | | |
| | Mapoly0157s0026.1 | -3.48 | 3.06E-19 | 33 | Peroxidase |
| | Mapoly0161s0001.1 | -3.38 | 1.06E-17 | 24 | Peroxidase |
| | Mapoly1415s0001.1 | -3.25 | 6.49E-18 | 28 | Xyloglucan endo-transglycosylase |
| | Mapoly0655s0002.1 | -3.16 | 1.10E-13 | 17 | Peroxidase |
| | Mapoly0352s0001.1 | -2.87 | 1.21E-35 | 143 | Cupin |
| | Mapoly0030s0038.1 | -2.82 | 4.30E-12 | 24 | Unknown |
| | Mapoly0006s0118.1 | -2.78 | 3.29E-24 | 54 | Lecton domain containing receptor kinase |
| | Mapoly0149s0004.1 | -2.73 | 4.95E-12 | 42 | Glycosyl hydrolase |
| | Mapoly0023s0089.1 | -2.64 | 3.84E-42 | 128 | Unknown |
| | Mapoly0022s0078.1 | -2.60 | 2.72E-14 | 44 | Aspartic protease family |
| | Mapoly0115s0006.1 | -2.53 | 1.58E-11 | 90 | Amino acid permease |
| | Mapoly0085s0059.1 | -2.46 | 1.31E-08 | 13 | Cupin |
| | Mapoly0145s0006.1 | -2.36 | 7.03E-16 | 226 | Polyphenol oxidase |
| | Mapoly0030s0037.1 | -2.27 | 3.38E-07 | 12 | Unknown |
| | Mapoly0157s0027.1 | -2.22 | 1.52E-06 | 7 | Peroxidase |
| | Mapoly0352s0002.1 | -2.21 | 2.10E-17 | 127 | Cupin |
| | Mapoly0006s0117.1 | -2.19 | 1.20E-17 | 82 | Unknown |
| | Mapoly0128s0035.1 | -2.17 | 9.37E-08 | 39 | Peroxidase |
| | Mapoly0109s0040.1 | -2.17 | 1.90E-09 | 565 | Sucrose phosphate synthase |
| | Mapoly0054s0122.1 | -2.14 | 2.73E-10 | 64 | Phosphate transporter family |
| | Mapoly0082s0048.1 | -2.14 | 3.17E-23 | 1029 | Unknown |

To determine how changes in the most highly up-regulated and down-regulated genes from each treatment may contribute to plant response, the top 100 most highly up and down regulated genes were taken and grouped by Gene Ontology (GO) function. Under 4 hours of high fluence treatment a large number of the total up-regulated genes are of unknown function (Fig. 5.1). The next highest group relates to oxidoreductase activity and this contributed 8% of measured genes. Protein binding, transferase activity and DNA binding regulation of transcription were the next three highest making up 5%, 5%, and 4% respectively. DNA repair also contributed marginally at 2% (Fig. 5.1)

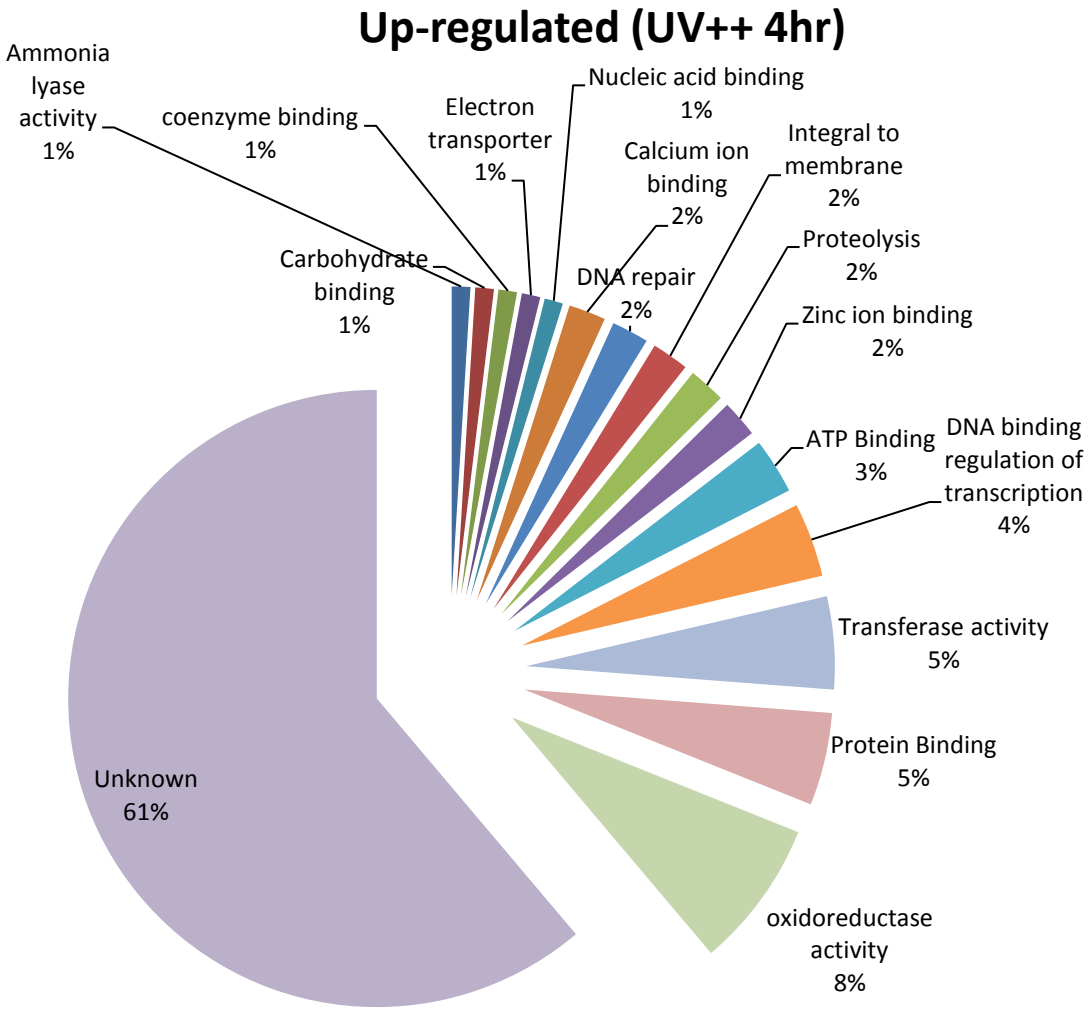



Figure 5.1: Top 100 genes up-regulated in high fluence treatments at 4 hours. Sorted by GO annotation category.

At 4 hours of high fluence UV-B a total of 46% of the top 100 down-regulated genes grouped by GO annotation are unknown (Fig. 5.2). Oxidoreductase activity accounted for 13% of those down regulated while hydrolase activity 6%, transferase activity 5%, membrane transport 4% and protein binding 4% make up the next largest groups of down regulated genes. DNA binding regulation of transcription makes up only 1% of down regulated genes as compared to 4% in the up-regulated group. A number of chlorophyllase activity genes were also found making up 3% and gene function contributing to nutrient reservoir activity made up 2% of the top 100 down regulated genes (Fig. 5.2)

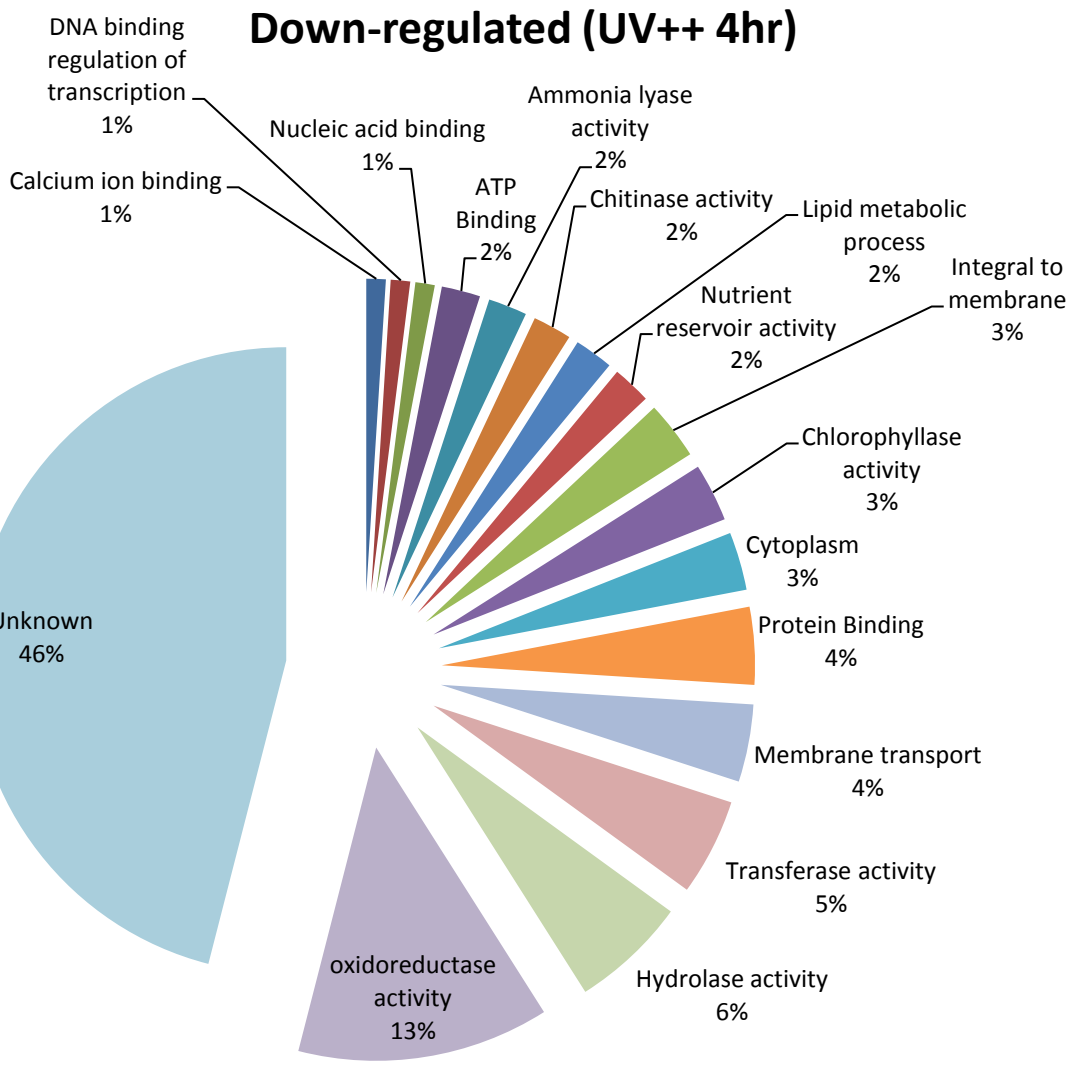



Figure 5.2: Top 100 genes down-regulated in high fluence treatments at 4 hours. Sorted by GO annotation category.

GO annotations were also used for the top 100 up and down regulated genes in the high fluence 1 day treatment. The highest group of up-regulated genes belonged to that of unknown function (55%) (Fig. 5.3). Oxidoreductase activity was again the next highest group making up 7% followed by protein binding 6%, ATP binding 6%, zinc ion binding 4% and DNA binding regulation of transcription 4%. Nutrient reservoir activity made up 3% of those up-regulated while DNA repair made up only 1% (Fig. 5.3).

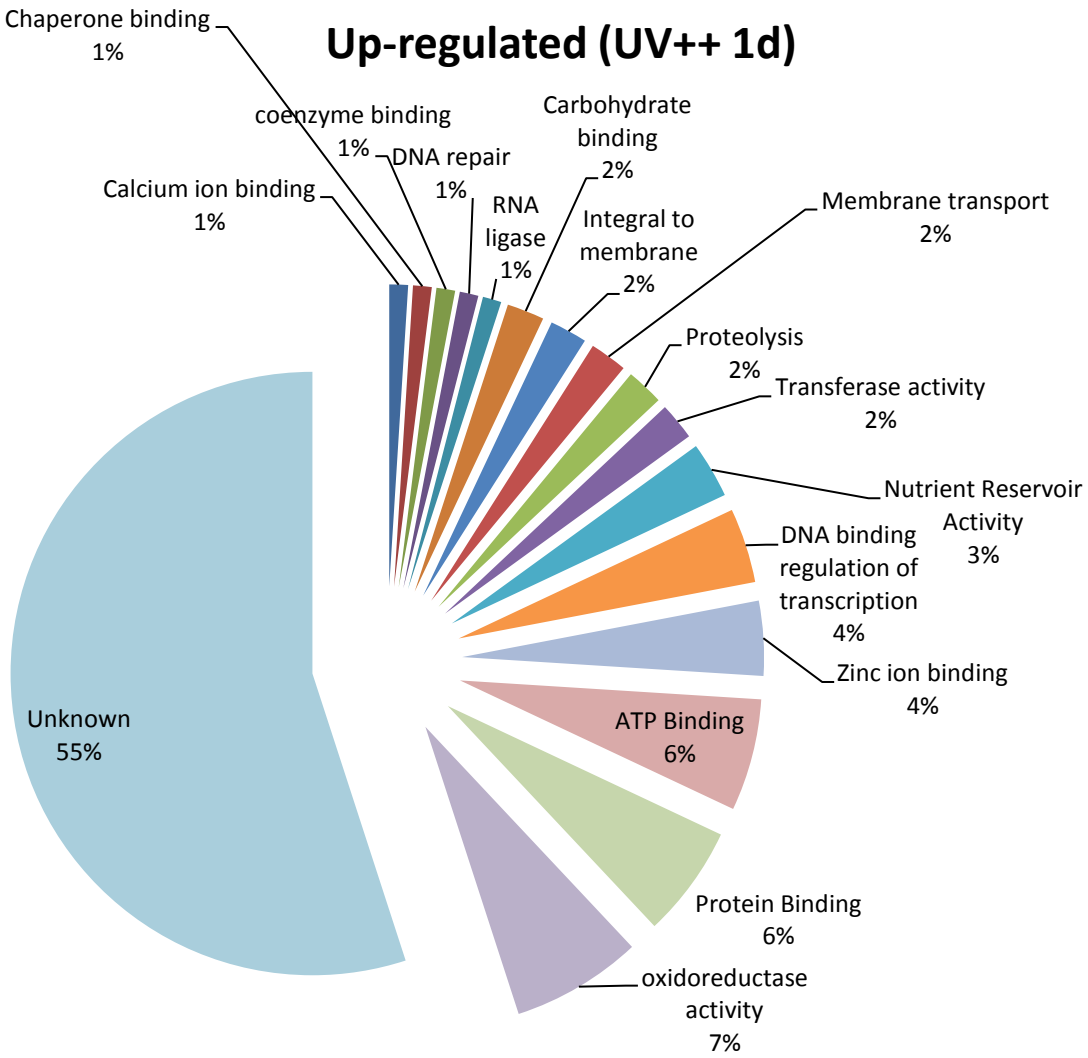



Figure 5.3: Top 100 genes up-regulated in high fluence treatments at 1 day. Sorted by GO annotation category.

Down regulated GO annotations of the high fluence treatment showed unknown gene functions made up 38% (Fig. 5.4). Membrane transport was the second highest group making up 21% and this was followed by oxidoreductase activity which made up 15%. Hydrolase activity functions contributed 7% while minor contributions were made by calcium ion binding (3%), transferase activity (3%) and cell wall organisation (2%) (Fig. 5.4).

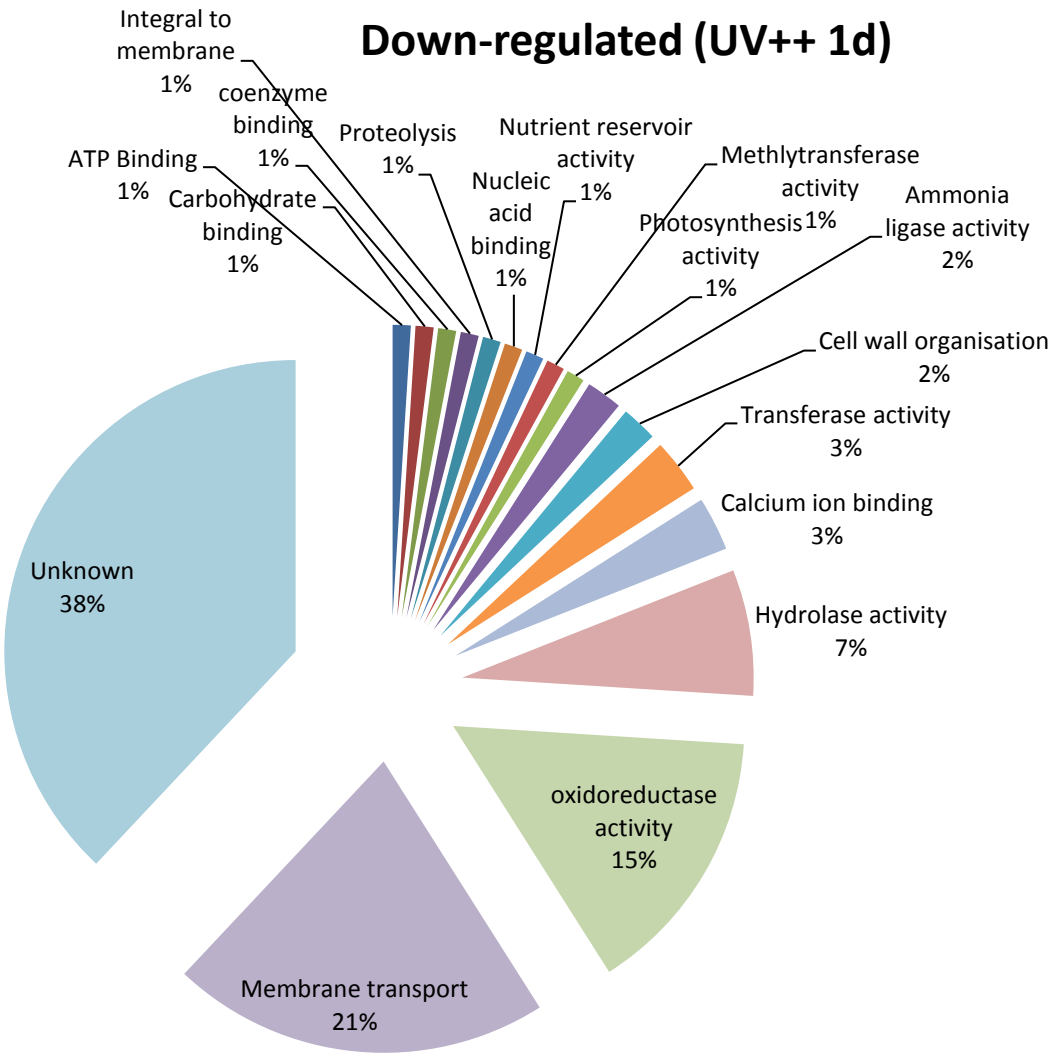



Figure 5.4: Top 100 genes down-regulated in high fluence treatments at 1 day. Sorted by GO annotation category.

Gene changes from plants that underwent low fluence treatment and sampled at 1 day were also analysed in the same way (Fig. 5.5, 5.6). Genes of unknown function made up 49% and oxidoreductase activity made up the second highest group at 18% of up-regulated genes (Figure 5.5). In both data sets oxidoreductase activity made up the second highest group after those of unknown function in the up-regulated data sets. Low fluence up-regulated genes had smaller contributions from metal ion transport (5%), membrane transport (4%), nucleic acid binding (4%), protein binding (3%) and nutrient reservoir activity (3%). Additionally a group making up 2% related to putative genes with functions of response to wounding (Fig. 5.5).

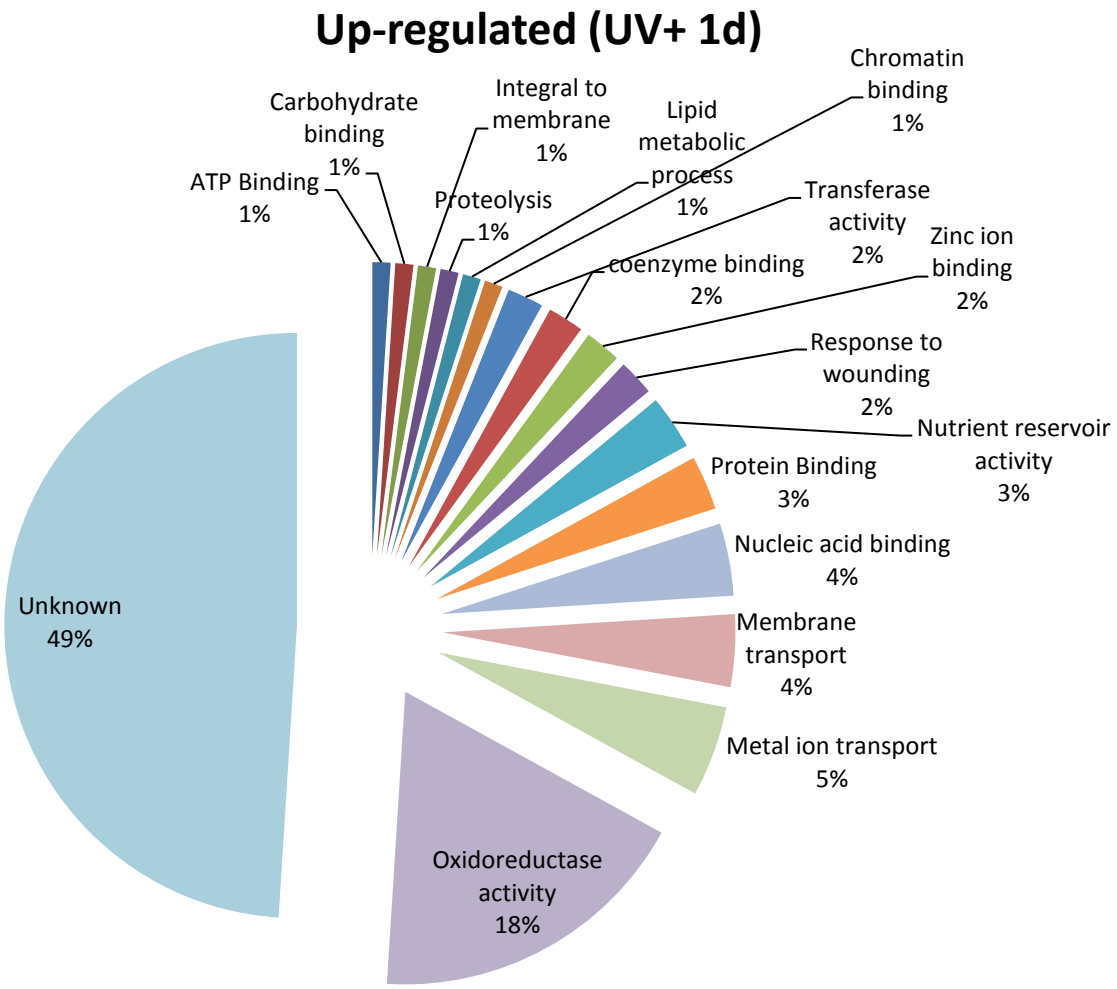



Figure 5.5: Top 100 genes up-regulated in low fluence treatments at 1 day. Sorted by GO annotation category.

The top 100 down-regulated genes under low fluence at 1 day showed unknown functions made up 38% (Fig. 5.6). Oxidoreductase activity contributed 17% while nutrient reservoir activity made up 9% of the top 100 down-regulated genes. Smaller groups were made up of functions related to integral to membrane (7%), transferase activity (5%), hydrolase activity (4%), cell wall organisation (3%) and membrane transport (3%) (Fig. 5.6).

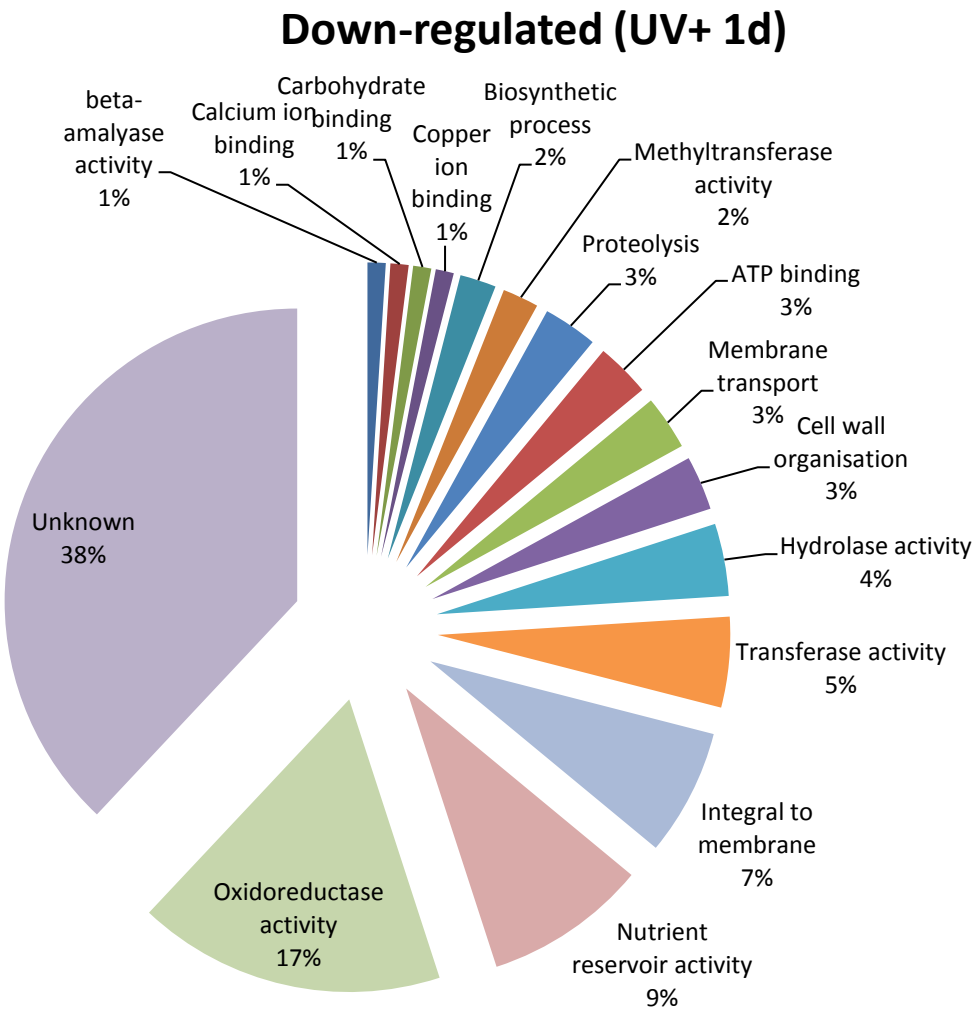



Figure 5.6: Top 100 genes down-regulated in low fluence treatments at 1 day. Sorted by GO annotation category.

### 5.2.2 Fold change regulation of putative gene families in *M. polymorpha* under UV-B conditions

To determine how important sets of gene families such as flavonoid regulators, DNA repair, peroxidases and general stress response families contribute to the UV-B response, gene models that had protein function for each relevant category were grouped and the fold change represented graphically.

To determine how the general flavonoid response may contribute to each treatment a group of putative genes from PAL, polyketide synthase, cytochrome p450, 2–oxoglutarate-dependent dioxygenase, UDP-glycosyltransferases and O-methyltransferases families were identified and the two-fold change under each treatment compared (Fig. 5.7). Low fluence models showed that a large number of genes were up regulated and of these around a third were up-regulated by over 1 log2fold change. This up-regulation was seen in both the high 4h and 1d treatments but a lesser number of total genes were up regulated under high fluence than low fluence (Fig. 5.7). Down regulated counts showed that low fluence treatment had smaller numbers of down-regulated genes as compared to high fluence treatments and that high fluence 4hr treatment had a larger number of strongly down-regulated (log2fold change > 1) than either other treatment.

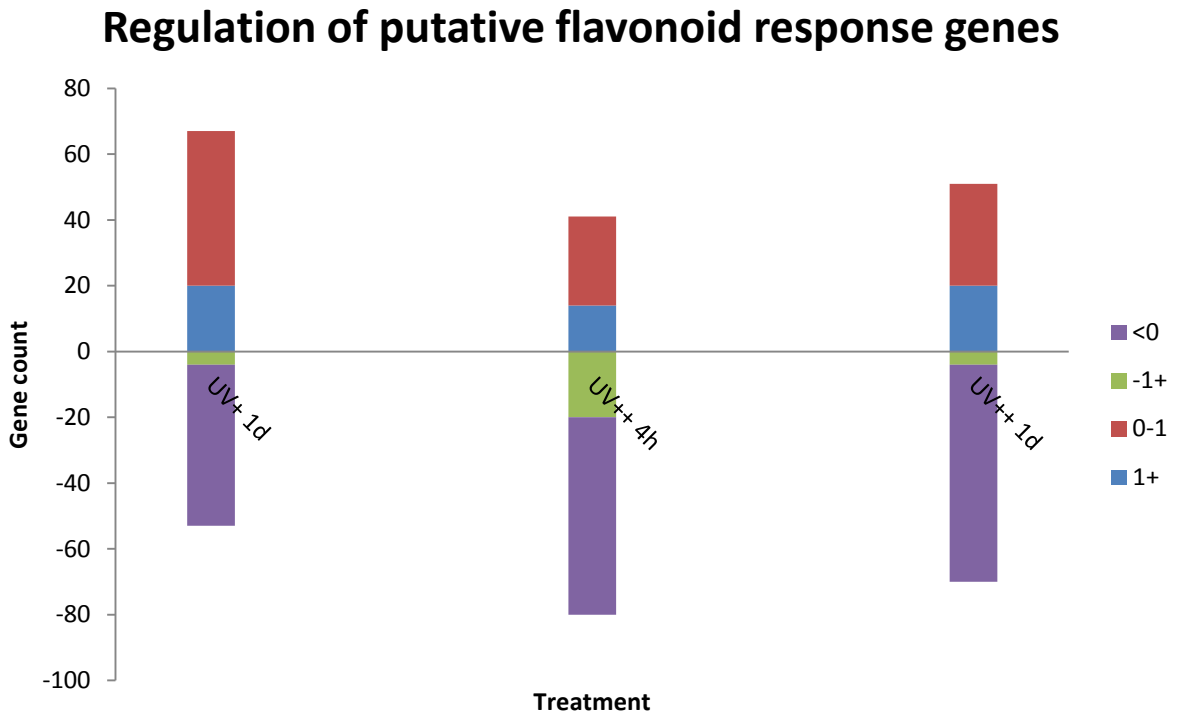



Figure 5.7: Regulation of flavonoid response genes under UV-B treatments. Putative genes from phenylalanine lyase, polyketide synthase, phenylpraponoid, cytochrome p450, 2–oxoglutarate-dependent dioxygenase, UDP-glycosyltransferases and O-methyltransferase families. Counts show up-regulated genes between 0 and 1, above 1, and down-regulated genes between 0 and 1, and below 1 log2fold change. Treatments were low fluence 1 day (UV+ 1d), high fluence 4 hours (UV++ 4h) and high fluence 1 day (UV++ 1d).

Peroxidase genes may function to mitigate ROS in plants that undergo UV-B treatment. A group of putative peroxidase genes was analysed and the fold change of up and down-regulated genes determined and counted under each treatment condition (Fig. 5.8). Low fluence treatment at 1 day had the highest number of up-regulated counts but it also had the highest number of strongly down-regulated counts that were above twofold change of 1. High fluence 4 hour treatment had a smaller amount of genes that were up-regulated but this increased at high fluence 1 day. The number of putative peroxidase genes down-regulated in the high 4 hour treatment was greater in both total and those strongly down-regulated than in that of the high 1 day treatment (Fig. 5.8).

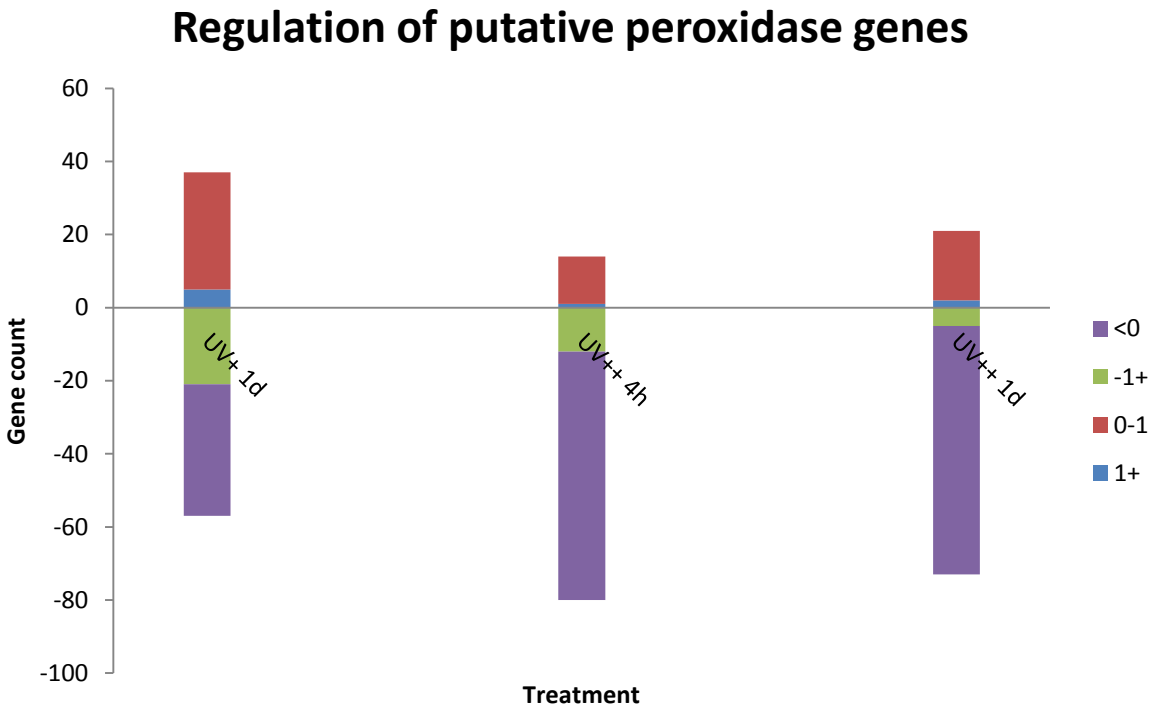



Figure 5.8: Regulation of putative peroxidase genes under UV-B treatments. Putative peroxidase genes grouped by two-fold change of each treatment. Counts show up-regulated genes between 0 and 1, above 1, and down-regulated genes between 0 and 1, and below 1 log2fold change. Treatments were low fluence 1 day (UV+ 1d), high fluence 4 hours (UV++ 4h) and high fluence 1 day (UV++ 1d).

Genes with putative functions relating to DNA repair were also counted and measured for twofold change (Fig. 5.9). The highest level of up-regulation was seen in the high fluence treatment at 1 day with the largest number of total and over 1 twofold change genes. Low fluence treatment also showed a large group that was up-regulated but did not have the same number of strongly up-regulated DNA repair genes as compared to the high fluence treatments. Correspondingly a larger amount of genes were down-regulated in the low fluence treatment but none were over twofold change of 1 (Fig. 5.9).

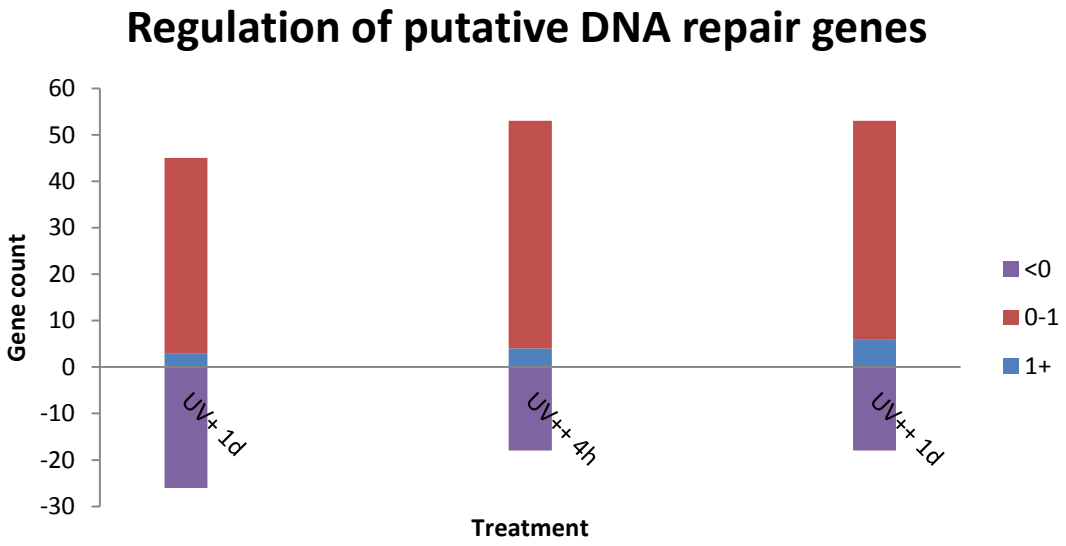



Figure 5.9: Regulation of putative DNA repair genes under UV-B treatments. Putative DNA repair genes grouped by two-fold change of each treatment. Counts show up-regulated genes between 0 and 1, above 1, and down-regulated genes between 0 and 1, and below 1 log2fold change. Treatments were low fluence 1 day (UV+ 1d), high fluence 4 hours (UV++ 4h) and high fluence 1 day (UV++ 1d).

Heat shock proteins have been found to be important in many stress responses and heat shock proteins were found to be highly up-regulated in the high fluence 1 day data set (Table 5.2). A group of putative genes related to heat shock proteins was analysed for the response under each of the treatment conditions (Fig. 5.10). High fluence 1 day treatment showed the highest amount of genes that were up-regulated and also the largest amount of strongly up-regulated genes over 1 twofold change. High fluence at 4 hours showed a smaller response with fewer genes up-regulated and a higher proportion down-regulated, but none were strongly down-regulated. Similarly the low fluence treatment showed a large number of up-regulated genes that related to heat shock proteins but in total numbers were lower than high fluence 1 day treatment (Fig. 5.10).

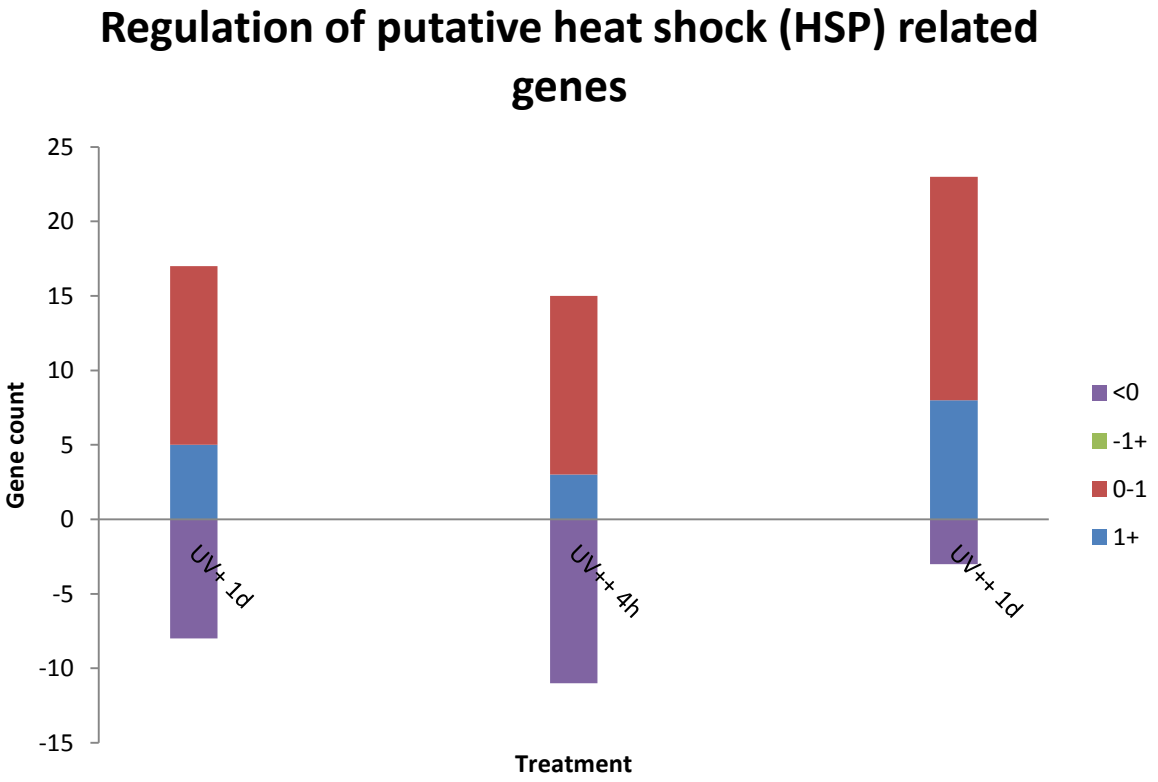



Figure 5.10: Regulation of putative heat shock genes under UV-B treatments. Putative heat shock genes grouped by two-fold change of each treatment. Counts show up-regulated genes between 0 and 1, above 1, and down-regulated genes between 0 and 1, and below 1 log2fold change. Treatments were low fluence 1 day (UV+ 1d), high fluence 4 hours (UV++ 4h) and high fluence 1 day (UV++ 1d).

Similarly chlorophyll A-B binding proteins contributed to a large number of genes up-regulated under high fluence 4 hour treatment, which corresponded to UV-B irradiance. A large group of chlorophyll A-B binding protein genes were analysed and the response determined under each treatment and compared (Fig. 5.11). High fluence at 4 hours showed the largest number of up-regulated genes and a large amount were up-regulated strongly. Comparatively low 1 day and high 1 day treatments showed small amounts of up-regulated genes and larger amounts of down-regulated genes (Fig. 5.11). Similarly genes that corresponded to the biosynthesis of chlorophyll were also measured in the same way (Fig. 5.12). Large numbers of up-regulated genes were seen in the high 4 hour and low 1 day treatments with the high fluence 4 hour samples showing the strongest response. High fluence 1 day treatment showed lower counts of up-regulated genes but the number of strongly up-regulated genes was similar to that of the other treatments. High fluence 1 day also had the highest count of down-regulated putative chlorophyll biosynthesis genes (Fig. 5.12).

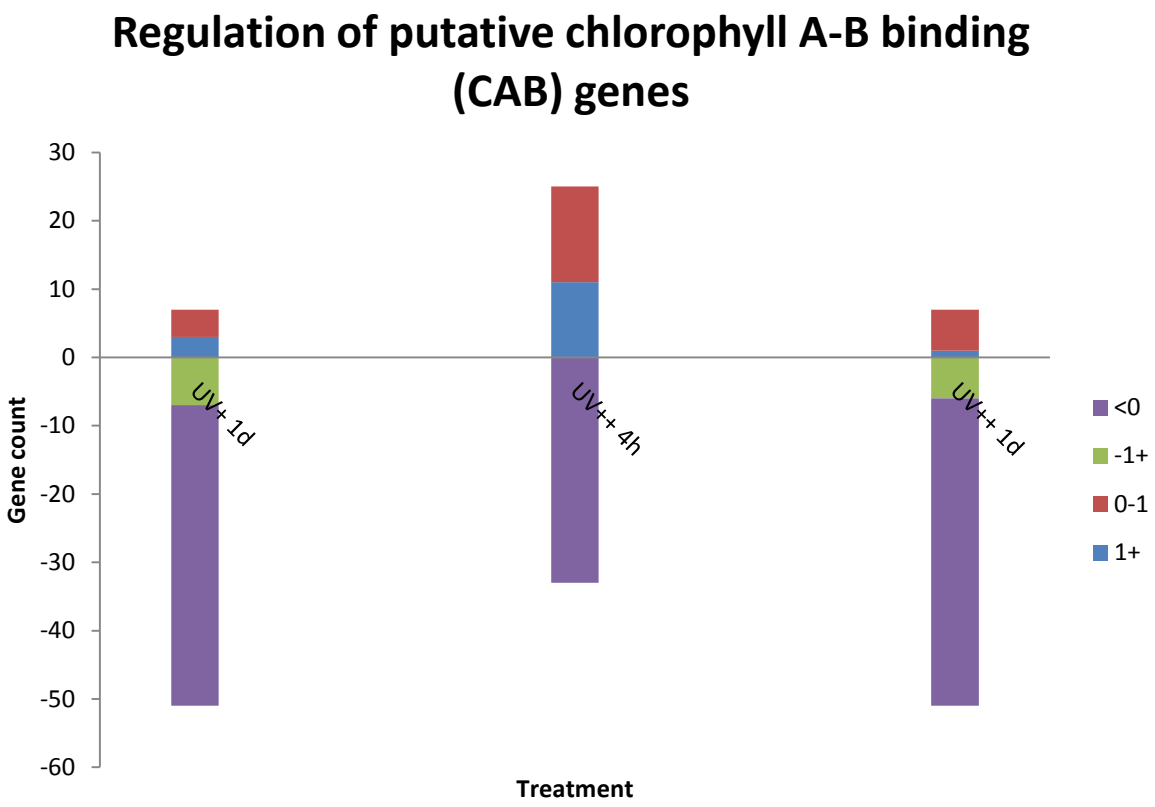



Figure 5.11: Regulation of putative chlorophyll A-B binding genes under UV-B treatments. Putative chlorophyll A-B binding genes grouped by two-fold change of each treatment. Counts show up-regulated genes between 0 and 1, above 1, and down-regulated genes between 0 and 1, and below 1 log2fold change. Treatments were low fluence 1 day (UV+ 1d), high fluence 4 hours (UV++ 4h) and high fluence 1 day (UV++ 1d).

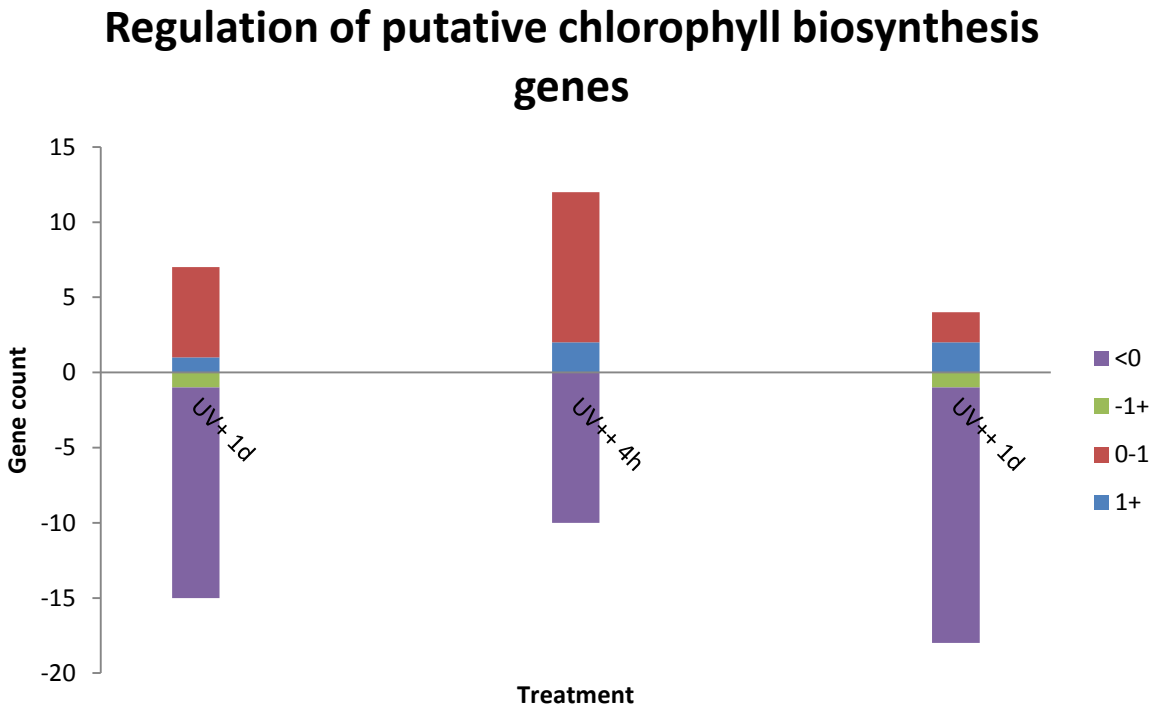



Figure 5.12: Regulation of putative chlorophyll biosynthesis genes under UV-B treatments. Putative chlorophyll biosynthesis genes grouped by two-fold change of each treatment. Counts show up-regulated genes between 0 and 1, above 1, and down-regulated genes between 0 and 1, and below 1 log2fold change. Treatments were low fluence 1 day (UV+ 1d), high fluence 4 hours (UV++ 4h) and high fluence 1 day (UV++ 1d).

A group of chitin binding genes were also analysed to determine their response under each treatment (Fig. 5.13). Putative chitin binding proteins were analysed as they are involved largely in pathogen response in higher plants and may indicate a stress response after UV-B irradiation in *M. polymorpha*. Chitin binding genes were found to be up-regulated at 1 day in both the high and low fluence treatments while they were heavily down regulated in the high fluence 4 hour treatment. Of the total down-regulated genes a large number were strongly down-regulated in the high fluence 4 hour treatment (Fig. 5.13).

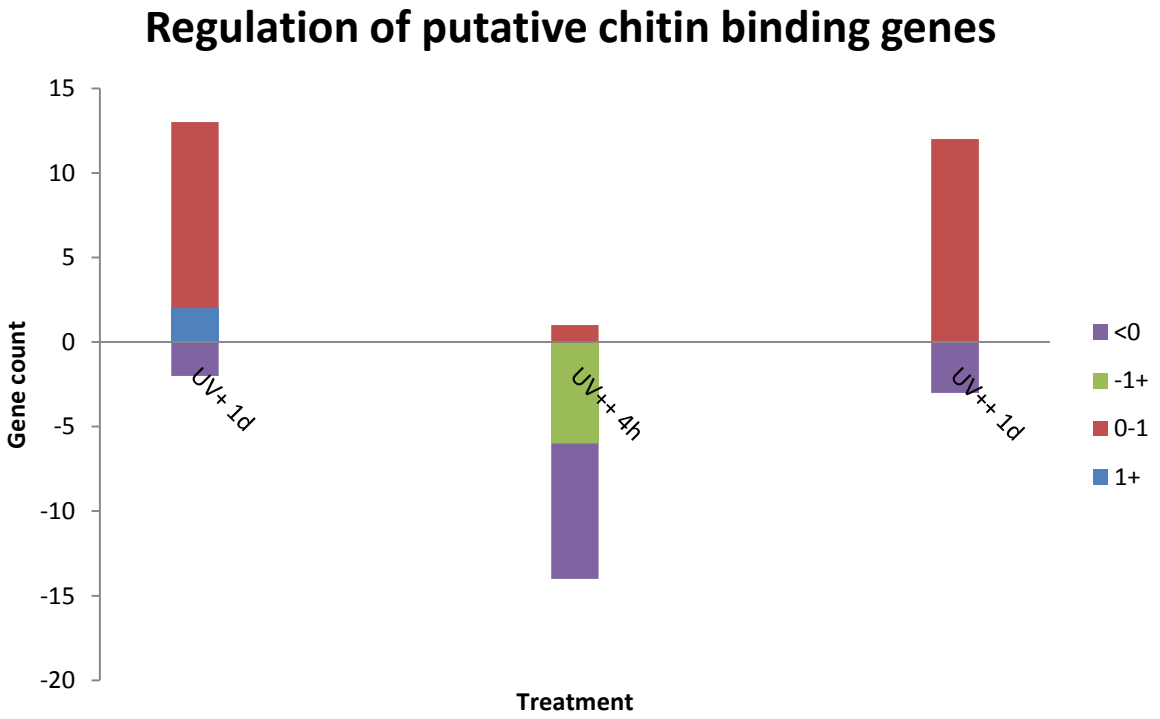



Figure 5.13: Regulation of putative chitin binding genes under UV-B treatments. Putative chitin binding genes grouped by two-fold change of each treatment. Counts show up-regulated genes between 0 and 1, above 1, and down-regulated genes between 0 and 1, and below 1 log2fold change. Treatments were low fluence 1 day (UV+ 1d), high fluence 4 hours (UV++ 4h) and high fluence 1 day (UV++ 1d)

Similarly genes involve in the ethylene response were also analysed to determine the effect of each treatment (Fig. 5.14). It was found that the largest number of up-regulated genes was under the high fluence 4 hour treatment but the number of strongly up-regulated genes was higher under the high and low 1 day treatments points. Correspondingly both high and low 1 day treatments had higher amounts of down-regulated genes although none of these were strongly down-regulated over twofold change of -1 (Fid. 5.14).

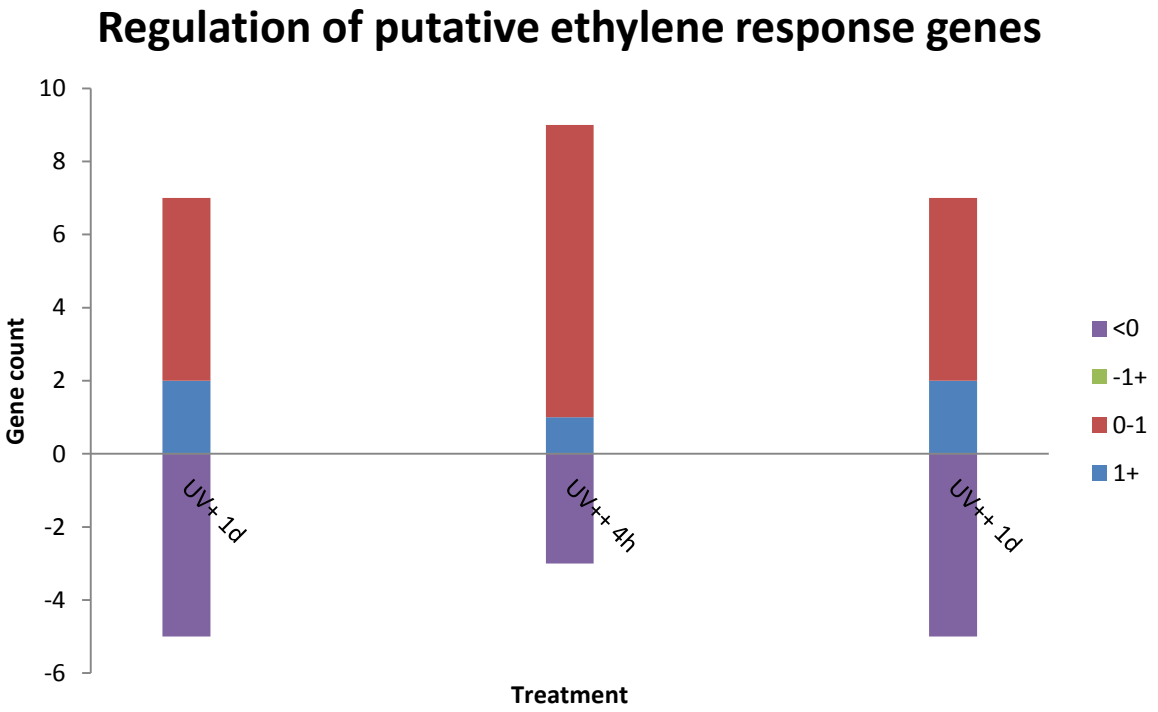



Figure 5.14: Regulation of putative ethylene response genes under UV-B treatments. Putative ethylene response genes grouped by two-fold change of each treatment. Counts show up-regulated genes between 0 and 1, above 1, and down-regulated genes between 0 and 1, and below 1 log2fold change. Treatments were low fluence 1 day (UV+ 1d), high fluence 4 hours (UV++ 4h) and high fluence 1 day (UV++ 1d)

### 5.2.3 Flavonoid regulator genes and biosynthetic gene changes in *M. polymorpha* under UV-B conditions

Under UV-B irradiance of high fluence at 4 hours it was seen that CHS, HY5 and RUP1 gene candidates were highly up-regulated. The synthesis of flavonoids can be mediated through the direct sensing of UV-B from UVR8 and signal transduction that results in the activation of flavonoid biosynthetic genes (Jordan, 2017). The major regulators of the UVR8 signal transduction were found and mapped for fold change (Fig. 5.16). Putative genes for COP1, RUP1 and HY5 were found to be up-regulated by 1.58, 3.84 and 3.38 log2fold respectively over the no UV-B control. A putative SPA gene (Mapoly0100s0059.1) was also up-regulated 0.6 fold under UV-B conditions. A UVR8 candidate was not up-regulated and showed minor down-regulation of -0.353 fold (Fig. 5.16).

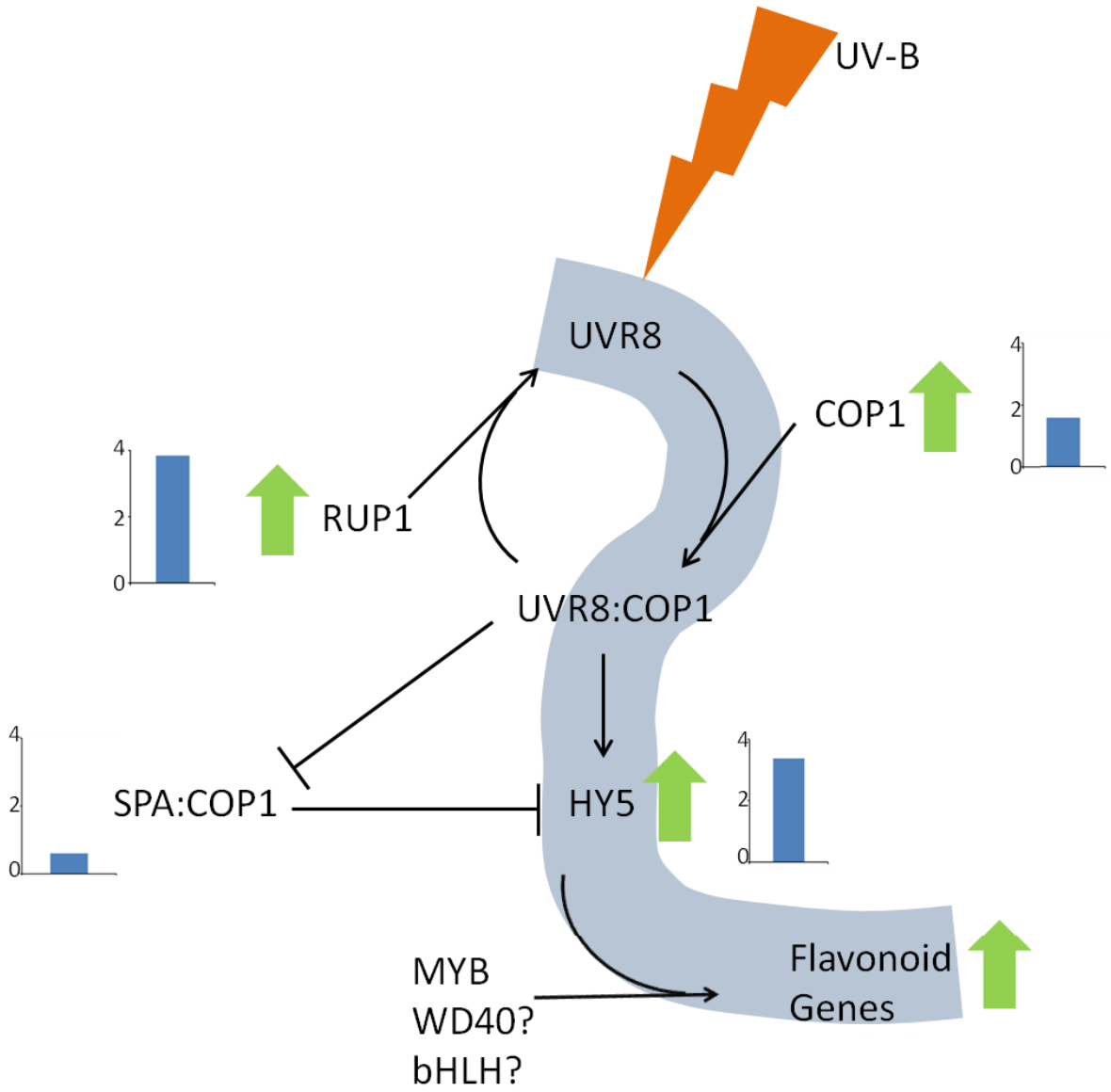



Figure 5.16: The specific UVR8 pathway and gene changes for key regulators that occur under 4 hours high UV-B. Graphs indicate log2fold change over a UV- control. Arrows indicate genes or group of genes that are up-regulated. Gene models; UVR8 (no significant change) (Mapoly0023s0125) RUP1 (Mapoly0094s0072.1), COP1 (Mapoly0143s0030.1), SPA (Mapoly0100s0059.1), HY5 (Mapoly0001s0021.1).

While the key regulators of the UVR8 pathway were seen to be up-regulated under UV-B conditions the biosynthetic genes that result in production of flavonoid compounds may also be regulated differentially under UV-B conditions. Candidate genes for biosynthetic genes were found and the fold change observed under UV-B high fluence conditions (Fig. 5.17). It

was found that key putative biosynthetic genes PAL, CHS, CHI, CHI-like, FNR and FNS were up-regulated under UV-B conditions. The candidate for PAL was up-regulated 2 log2fold, CHS 3, CHI 0.6, CHI-L 1.7, FNS 2.36 and FNR 1.36 over the non UV-B control (Fig. 5.17). Candidates for FNS and FNR are putative assignments.

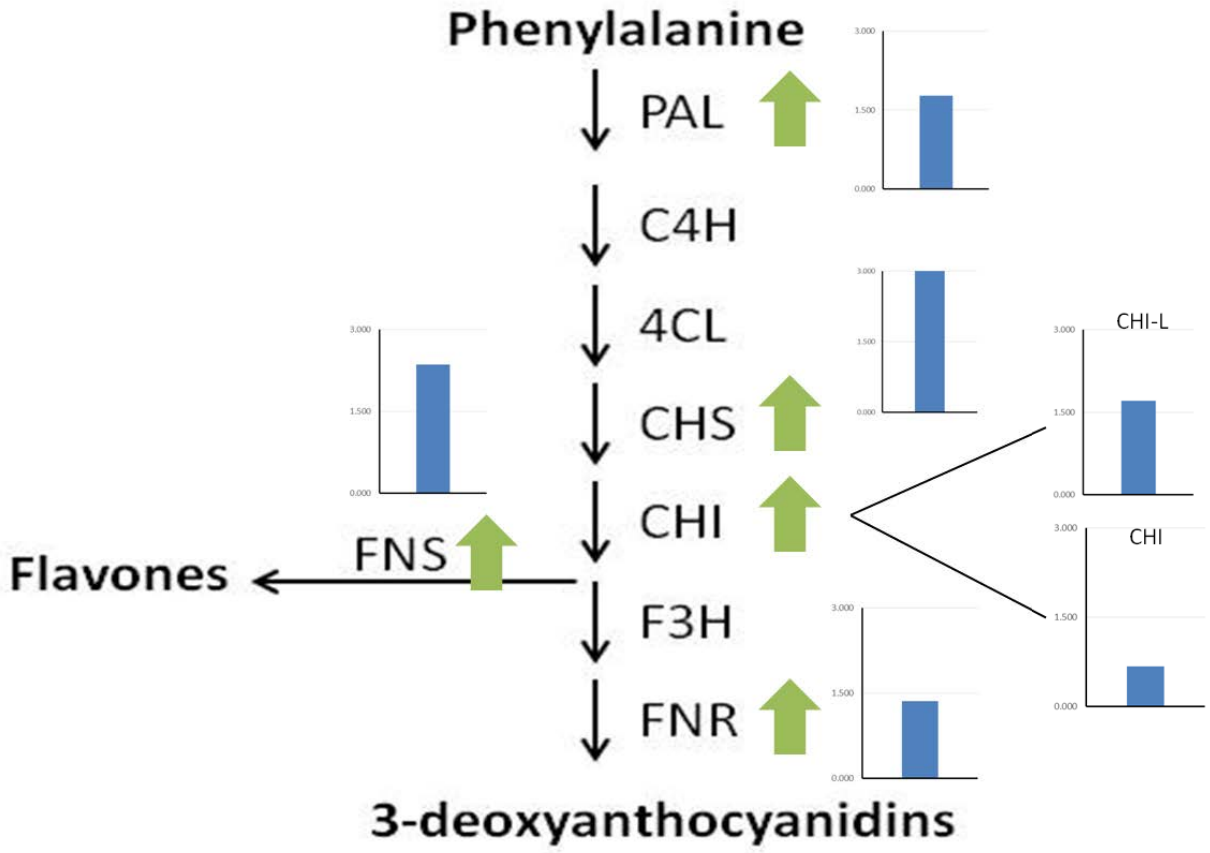



Figure 5.17: The specific flavonoid pathway in *M. polymorpha* and the key biosynthetic genes regulated under 4 hours of high UV-B conditions. Graphs indicate two fold change over a UV-control. Arrows indicate putative genes that are up-regulated. Gene models; PAL (Mapoly0132s0049.1), CHS (Mapoly0021s0159.1), CHI (Mapoly0167s0012.1), CHI-L (Mapoly0175s0004.1), FNS (Mapoly0002s0224.1), FNR (Mapoly0063s0048.1).

Genes of interest for the UV response in *M. polymorpha* were also found. For each transcript the response under each treatment condition was shown (Table 5.4). Under high fluence 4 hours (UV-B irradiation) the gene response for SPA1, COP1, RUP1 and HY5 are up-regulated as previously outlined (Fig. 5.16). Many of these genes while highly up-regulated under UV-B

at 4 hours dropped dramatically when UV-B was removed at 1 day although RUP1 was still highly up-regulated in high fluence treatment at 1 day and less so in low fluence at 1 day (Table 5.4). Likewise HY5 was still up-regulated in high fluence plants at 1 day and less so in low fluence 1 day plants, yet both were much less than under UV-B at 4 hours high fluence. As well as these genes an early light induced protein (ELIP), DNA repair, and photosystem II protein D1 were also up-regulated under UV-B light at 4 hours. The response at 1 day low and high fluence showed up-regulation of a short under blue light (SUB1) homologue, MYB14 transcription factor, GLABRA3 (GL3), WRKY interacting protein, heat shock proteins (HSP), metallochaperone, peroxidase, DNAJ chaperone and PSII protein D1 genes. An ERF was found to be up-regulated strongly at high fluence 4 hours and 1 day but was not differentially expressed in the low fluence treatment data set at 1 day.

Genes for UVR8, GL3 and a peroxidase were down-regulated under UV-B irradiance at 4 hours of high fluence (Table 5.4). UVR8 was also down-regulated in the high fluence 1 day treatment and the gene model was not differentially expressed in the low fluence data set. At 1 day in both high and low fluence treatments a phytoene synthase and RUBISCO small subunit gene were down-regulated. SPA1 was also seen to be down-regulated slightly in the high fluence 1 day treatment.

Table 5.4: Differentially expressed genes of *M. polymorpha* under UV-B treatment. Genes of interest relating to UV response, transcription and stress are shown with log two fold change as related to a UV-B lacking control. Maximum base mean for each gene is shown along with the gene transcript model number. – Indicates lack of significant differential expression in transcript data. Low fluence 1 day (1d LF), high fluence 4 hour (4h HF) and 1 day high fluence (1d HF) are shown.

| Pathway | Gene | 1d LF log2 | 4h HF log2 | 1d HF log2 | Max Base mean | Mpoly_primaryTS |
|---|---|---|---|---|---|---|
| UV Response | SPA1 | - | 0.59 | -0.1 | 1282 | Mapoly0100s0059.1 |
| UV Response | COP1 | - | 1.58 | 0.09 | 1482 | Mapoly0143s0030.1 |
| UV Response | RUP1 | 0.72 | 3.84 | 2.20 | 1788 | Mapoly0094s0072.1 |
| UV Response | UVR8 | - | -0.35 | -0.53 | 1260 | Mapoly0023s0125.1 |
| UV Response | ELIP1 | - | 3.81 | 2.96 | 3564 | Mapoly0047s0106.1 |
| UV Response | SUB1 | 1.73 | 0.21 | 1.68 | 1697 | Mapoly0016s0050.1 |
| Transcription | HY5 | 0.45 | 3.38 | 1.06 | 908 | Mapoly0001s0021.1 |
| Transcription | MYB14 | 2.66 | 0.16 | 2.92 | 193 | Mapoly0073s0038.1 |
| Transcription | GL3 | 3.60 | -0.71 | 2.59 | 550 | Mapoly0028s0058.1 |
| Transcription | WRKY Interacting | 3.42 | 2.29 | 4.69 | 524 | Mapoly0044s0085.1 |
| Transcription | ERF | - | 2.41 | 5.66 | 160 | Mapoly0166s0010.1 |
| Flavonoid Biosynthesis | PAL | -0.37 | 1.77 | 0.78 | 2816 | Mapoly0014s0211.1 |
| Flavonoid Biosynthesis | CHS | -0.28 | 3.01 | 1.18 | 7186 | Mapoly0021s0159.1 |
| Flavonoid Biosynthesis | CHI | -0.39 | 0.67 | -0.04 | 783 | Mapoly0167s0012.1 |
| Flavonoid Biosynthesis | CHI-L | 0.58 | 1.70 | 0.87 | 1385 | Mapoly0175s0004.1 |
| Flavonoid Biosynthesis | FNS | -0.44 | 2.36 | 0.29 | 5144 | Mapoly0002s0224.1 |
| Flavonoid Biosynthesis | FNR | 1.19 | 1.36 | 1.39 | 3465 | Mapoly0063s0048.1 |
| UVB stress | HSP70 | 0.79 | 0.85 | 1.55 | 7227 | Mapoly0013s0060.1 |
| UVB stress | HSP18 | 1.99 | 0.43 | 6.20 | 183 | Mapoly0076s0006.1 |
| UVB stress | DNA Glycoslyase | 0.55 | 3.58 | 1.54 | 309 | Mapoly0074s0054.1 |
| UVB stress | DNA Photolyase | 1.11 | 2.21 | 1.42 | 440 | Mapoly0002s0311.1 |
| UVB stress | DNA Photolyase | -0.03 | 1.43 | 0.18 | 626 | Mapoly0149s0026.1 |
| UVB stress | Metallochaperone | 4.04 | 4.39 | 5.25 | 2047 | Mapoly0025s0089.1 |
| UVB stress | Phytoene synthase | -0.88 | 0.05 | -0.94 | 2625 | Mapoly0040s0104.1 |
| UVB stress | RUBISCOssu | -1.45 | 0.55 | -1.08 | 1697 | Mapoly0114s0053.1 |
| UVB stress | Peroxidase | 4.37 | -0.20 | 2.04 | 67 | Mapoly0106s0052.1 |
| UVB stress | DNAJ Chaperone | 1.26 | 0.90 | 1.64 | 813 | Mapoly0088s0065.1 |
| UVB stress | PSII protein D1 | 1.74 | 2.04 | 2.16 | 352 | Mapoly0093s0016.1 |

To understand the synthesis of flavone compounds over time in both high and low fluence treatments as compared to the UV-B lacking control, treated plants were sampled and the RNA extracted for qRT-PCR. Transcript abundance was normalised to an actin control (Fig. 5.18). Relative abundance was seen to increase from 4 hours in both high and low fluence treatments and reached a peak at 12 hours. High fluence treatment was seen to induce the largest increase in mRNA abundance at 12 hours. At 15 hours transcript abundance decreased to untreated control in low fluence plants but remained elevated in high fluence plants. Twenty-eight hours post treatment transcript abundance in low fluence plants returned to steady state levels and remained at this level at 76 and 100 hours post initial UV-B treatment. High fluence transcripts were reduced at 76 and 100 hours as compared to the UV-B lacking control.

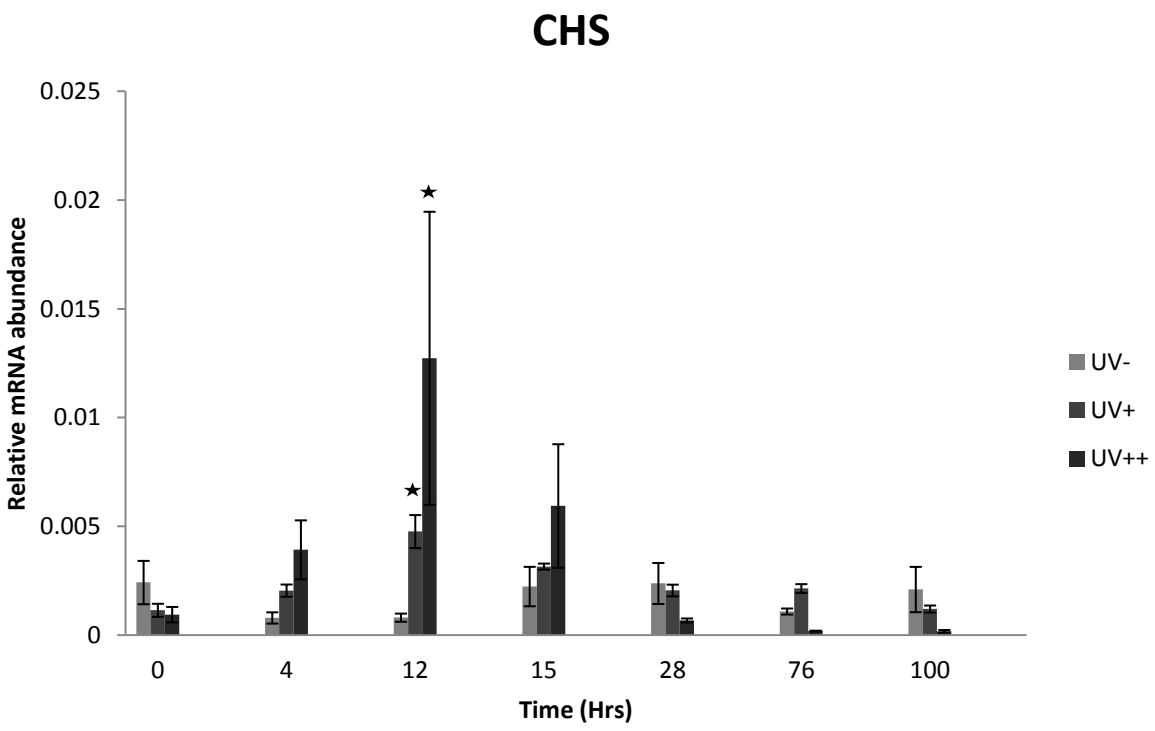



Figure 5.18: Relative expression of CHS with UV-B treatment. Transcript abundance for CHS is shown for control (UV-), low fluence (UV+) and high fluence (UV+) treatments over time. Relative transcript abundance was determined by qRT-PCR, normalised to an actin control. Error bars = standard error (n=3). (*$P$<0.05)

## 5.3 Discussion

### 5.3.1 RNA sequencing of *M. polymorpha* under UV-B conditions

RNA-seq analysis revealed large changes in the gene expression profile under different UV-B treatments. Under direct UV-B at 4 hours of high fluence the most up-regulated genes contained candidates for RUP1, HY5 and CHS genes which indicated a large response through the UVR8 mediated pathway for the induction of flavonoids. The highest up-regulated gene was that of a copper binding metallochaperone. Copper homeostasis is important in the mitochondria for the assembly of cytochrome c oxidase (COX) and also regulating the redox homeostasis due to metal induced ROS production (Garcia *et al.*, 2014). The putative COX assembly factor copper chaperone HCC2, has been described to be involved in the UV-B response in Arabidopsis (Steinebrunner *et al.*, 2014). However the loss of HCC2 had no effect on COX assembly and HCC2 had lost the copper binding domain suggesting it had a function other than copper binding. This function was postulated to be involved in oxidoreductase status in the mitochondria (Steinebrunner et al., 2014). The copper chaperone observed in our data does not have homology to HCC2 and its function is unknown. Importantly it was the only gene we found to be in the top up-regulated transcripts in each data set which may indicate its importance in the UV-B response, potentially through mediating ROS in the mitochondria.

Interestingly there was an ATP-binding cassette (ABC) family transporter highly up-regulated under high UV-B at 4 hours. ABC transporters have been found to be involved with the transport of flavonoids within plant tissues (Yazaki, 2006, Alfenito *et al.*, 1998). This gene may have a putative function for the transport of flavonoids to the vacuoles and its up-regulation under UV-B relates to the induction of flavonoid production we see, and thus the need for increased transport of these flavonoids. A putative DNA glycosylase was also found to be highly up-regulated under UV-B which may help protect DNA against UV-B damage (Sinha & Häder, 2002). UV induced DNA damage is a regular unwanted occurrence under UV-B and the repair mechanisms are well understood (Sinha & Häder, 2002). The DNA glycosylase up-regulation together with two putative DNA photolyase genes that are up-

regulated (Table 5.4) indicates protective measures against DNA damage are induced early in the UV-B response (4 hours). The up-regulation of these DNA repair genes continued at 1 day in the high fluence treatment while low fluence treatment at one day showed some up-regulation but this was very much reduced which suggested DNA damage under high fluence was much higher than under low fluence. Grouping all putative DNA repair genes together and looking at the fold change regulation (Fig. 5.9) it was also seen that overall the up-regulation of these genes was higher in both 4 hour and 1 day high fluence as compared to low fluence 1 day. This indicates that while the repair of DNA damage is important in both high and low fluence treatments, high fluence UV-B causes higher DNA damage due to increased UV-B irradiance and this damage must be repaired for plant function to continue.

Under UV-B high fluence for 4 hours chlorophyll A-B binding (CAB) proteins were found to be highly induced. One of these was found to be similar to that of an early light induced protein (ELIP) (Mapoly0047s00106.1). This was highly up-regulated under high fluence at 4 hours and also at 1 day of high UV-B fluence, while the gene model was not differentially expressed in the low fluence data set (Table 5.4). Total analysis of CAB proteins showed that while under UV-B at 4 hours many genes were up-regulated but at 1 day in both low and high fluence treatments expression was largely down-regulated (Fig. 5.11). This change in expression may largely be due to the increased fluence from UV-B which may induce the expression of CAB proteins for light harvesting or in the case of genes belonging to the ELIP family they may have protective function. ELIPs have been proposed to have protective function, through the binding of chlorophylls released during turnover of pigment-binding proteins or the stabilization of the proper assembly of those proteins, during high-light stress (Hutin *et al.*, 2003). In particular ELIPs have also been found to be induced by UV-B light (Hayami *et al.*, 2015, Adamska *et al.*, 1992, Sävenstrand *et al.*, 2004). Interestingly an element in ELIP2 that is induced in Arabidopsis under UV-B conditions was found to be able to bind HY5 *in vitro* suggesting its involvement in UV-B stress in a HY5 dependent manner. In much the same way high light stress may induce ELIP through HY5 by up-regulation through the cryptochrome pathway (Hayami et al., 2015). In this study we see the high induction of ELIP under UV-B conditions which likely serves in a protective function, potentially regulated through HY5 by UVR8, reducing damage to the photosynthetic machinery.

Gene regulation at one day is important to understand the stress response occuring after high and low fluence UV-B treatments. High fluence treated plants at one day showed the highest up-regulation corresponding to a heat shock protein (Mapoly0076s0006.1). Heat shock proteins help newly synthesised proteins achieve their native fold, which is particularly important under stress conditions (Niforou *et al.*, 2014, Jacob *et al.*, 2017). Oxidative stress may contribute to the misfolding of proteins by interacting with amino acids. In particular cysteine and methionine residues can be converted to disulfides and methionine sulfoxide residues, respectively (Jacob et al., 2017). As ROS have previously been seen to accumulate in high amounts in *M. polymorpha* high fluence treatments (Chapter 3), such interaction and misfolding may be problematic. The induction of heat shock proteins may help reduce damaging effects of ROS on protein function under high UV-B in *M. polymorpha*. The MAPK indirect pathway is largely induced under high UV-B conditions and involves MPK3 and MPK6 (González Besteiro et al., 2011). This pathway has also been shown to be activated in response to oxidative stress, similar to that caused by UV-B, and this activation directly lead to transcriptional activation of a heat shock protein in a MPK3 and MPK6 dependent manner (Pérez-Salamó *et al.*, 2014). Over-expression of heat shock protein in rice also caused higher resistance to UV-B stress, also proposed to be due to reducing the ROS response (Murakami *et al.*, 2004a). Grouping heat shock proteins induced in *M. polymorpha* (Fig 5.10) found that the highest up-regulation was seen in the high fluence 1 day treatment while up-regulation was also present, although at lower levels, in the low fluence treatment. This would indicate that heat shock proteins are required more for the high fluence treatments that could induce larger damage and in particular a larger ROS burden than in the low fluence treatment. Overall as seen in our high fluence data, high UV-B in *M. polymorpha* may induce the expression of heat shock proteins, likely through the indirect MAPK pathway and ROS, in order to reduce protein misfolding and continue cellular functioning.

Putative genes that were shared between the high and low 1 day treatments included a signal transduction adapter molecule, glutamine repeat protein, Na P-type ATPase, EamA-like transporter family, tRNA-splicing ligase RtcB, leucine rich repeat protein kinase, L-type lectin domain containing receptor kinase, germin-like protein, pectin esterase, heat shock

protein mitochondrial-like and a multicopper oxidase (Table 5.2, 5.3). The exact function of each is speculative but the shared up-regulation between high and low UV-B treatments suggests an importance for the response to UV-B itself. The heat shock protein mitochondrial-like may function in the mitochondria for the reduction of ROS affected missfolding as previously described. Multicopper oxidases include the laccase enzymes which are involved in the oxidation of flavonoid compounds (Pourcel *et al.*, 2007). In addition to this multicopper oxidase a polyphenol oxidase, which may also be involved in flavonoid oxidation, was also highly up-regulated in the high fluence data set at 1 day. This may indicate an important function of these oxidases in the response of *M. polymorpha* to UV-B, particularly high fluence UV-B. Flavonoids can be oxidised to produce their corresponding semiquinones and quinones which can react with phenols, amino acids or proteins to produce a complex mix of brown pigments (Pourcel et al., 2007). The up-regulation of these genes may somewhat explain the browning phenotype we see in high fluence treated *M. polymorpha* as the products of oxidised flavonoids react to produce brown pigments (chapter 3). The oxidation of flavonoids has been proposed to have functions in abiotic stress resistance including to that of UV-B potentially through the scavenging of ROS (Jansen *et al.*, 2001). The activity of multicopper oxidase and polyphenol oxidase we see in *M. polymorpha* may be caused by a need to reduce ROS produced in response to UV-B.

Low fluence at 1 day showed similar gene regulation to that of the high fluence 1 day data set but was also enriched for copper chaperone and peroxidase genes. Putative copper chaperones were found to be the top two up-regulated genes and a further two copper chaperone genes were also found to be within the top-20 up-regulated genes. Copper chaperone function has been discussed earlier and the high up-regulation of these genes may contribute to regulating ROS homeostasis in the cell and particularly within the chloroplast.

While genes with the largest differential may give an understanding of the specific regulation of some genes, the functional significance of large groups of highly up and down-regulated genes may be missed. In order to better understand the function of the highest up and down

regulated genes the top 100 up and down regulated genes were grouped by GO function and total counts plotted for comparison. After unknown function, oxidoreductase function contributed the highest number of up and down regulated genes in all data sets except for the high fluence 1 day down-regulated genes, where membrane transporters had a higher count. Oxidoreductase activity and the regulation of ROS homeostasis must therefore be an important factor in the response to UV-B at high and low fluence. The count of highly up-regulated genes that had this function in high fluence plants was similar in both 4 hour and 1 day data sets with 8, 7% up and 13, 15% down respectively. However the low fluence 1 day data differed strongly with 18% up and 17% down contributed from oxidoreductase activity. Interestingly we see a strong ROS accumulation in high fluence plants that we do not see in low fluence plants (Chapter 3), so this difference in accumulation may be due to the higher up-regulation of oxidoreductase genes in low fluence plants. ROS may be rapidly turned over by this increased gene regulation in low fluence plants while high fluence plants accumulate ROS at higher levels due to lower counts of up-regulation genes with oxidoreductase function. The higher number of down regulated genes in the low fluence data may be in order to balance the response and create an equilibrium of gene regulation in order to correctly respond to ROS levels. High fluence plants at 1 day had a higher count of membrane transport genes that were down-regulated. While the exact function the down-regulation of these genes may play in the UV-B response to high fluence is speculative, membranes are a target for UV-B and may be damaged by high UV-B fluence (Murphy, 1983). Such decrease in membrane transport may be a reflection of cellular stress and corresponding down-regulation of transport in favour of more protective measures. A significant change in the number of genes involved in nutrient reservoir activity was also observed in down-regulated genes at 1 day of low fluence UV-B. Nutrient reservoir activity relates to the storage of nutrients and the down-regulation of such function may be due to the need for utilising these nutrients. In our case this may be due to UV-B acclimation process directing nutrients away from storage and into the acclimatisation response, in particular the biosynthesis of flavonoid compounds.

### 5.3.2 Fold change regulation of putative gene families in *M. polymorpha* under UV-B conditions

Genes that corresponded to the protein families of the flavonoid response, peroxidase, DNA repair, heat shock, chlorophyll A-B binding (CAB), chlorophyll biosynthesis, chitin binding and ethylene response were measured for their up and down regulation in each treatment. The flavonoid response genes which contained a very broad range of biosynthetic candidates for the production of flavonoids showed the largest up-regulation in the low fluence 1 day treatment. High fluence 1 day treatment in comparison did not show the same number of total up-regulated genes. The number of highly up-regulated genes between low and high fluence treatments at one day was similar which may indicated that although more genes are up-regulated in low fluence a core set of flavonoid response genes that are highly regulated in response to UV-B is shared between both the high and the low fluence treatments. High fluence 4 hour treatment showed a larger count in down-regulated genes and this is most likely due to the 4 hour time point corresponding to early activation of the flavonoid response whereby many genes may later switch from down to up regulation to provide flavonoid biosynthesis in response to UV-B. This group included putative genes for phenylalanine ammonia lyase, polyketide synthase, phenylpropanoid, cytochrome p450, 2–oxoglutarate-dependent dioxygenase, UDP-glycosyltransferases and *0*-methyltransferases. While many candidates were analysed there may only be a core set of genes that respond specifically to UV-B and increase flavonoids. While the analysis of the whole group gives a broad view of the flavonoid response the key candidates for the flavonoid biosynthetic genes and regulators of the pathway are discussed later in this chapter.

A group of putative peroxidase genes were also analysed for each treatment condition. Peroxidase genes are involved in auxin metabolism, lignin and suberin formation, cross-linking of cell wall components, phytoalexin synthesis, and the metabolism of ROS and reactive nitrogen species (RNS) (Almagro *et al.*, 2009). As UV-B irradiance induces the formation of ROS the function of peroxidase genes in ROS reduction may be particularly important. The group analysed showed the highest number of genes up-regulated in the low fluence 1 day treatment as compared to high 4 hr and 1 day. However low fluence treated plants also showed higher counts of strongly down-regulated genes and may suggest that a balance between up and down-regulation occurs in low fluence plants to regulate the ROS

homeostasis. This potential balance was not seen in high fluence treatments with all genes largely down-regulated. This was not expected as high fluence plants are observed to have large increases in ROS and the detoxification of this would be important for plant function. As peroxidase genes may function in many different pathways the potential exists that the putative peroxidase genes analysed here may include just a few that are used for ROS detoxification. A flavonoid-peroxidase system for the detoxification of hydrogen peroxide has also been proposed (Yamasaki *et al.*, 1997). In brief, flavonoids could act as electron donors scavenging hydrogen peroxide, after which reaction with ascorbate may return flavonoids to their non-oxidised forms to complete the cycle. Such functions further strengthen the important role flavonoids may play in regulating ROS in response to UV-B.

DNA repair and heat shock proteins were found to be up-regulated, especially in the high fluence data sets. DNA is especially sensitive to UV-B through its absorption and the corresponding damage that occurs due to this. DNA repair genes would be especially important to regulate this damage. As such a group of DNA repair genes was analysed and showed that all treatments had up-regulation of these genes, although high fluence treatments had higher levels than low fluence. The increase in all treatments show the importance of reducing potential DNA damage. It must be noted that sampling at 1 day for both high and low fluence does not give any indication for the potential of flavonoid screening compounds to reduce the incident effects of UV-B on DNA damage as screening compounds may not have been produced to sufficient levels at this time point. A much better evaluation would have been to analyse acclimatised plants that contained high accumulation of flavonoids for the response in DNA damage. Acclimatised plants may show less damage due to UV-B screening flavonoids reducing UV-B entering the cell and being absorbed by DNA. Studies have shown in higher plants that indeed flavonoid compounds and the screening of UV-B reduces DNA damage (Kootstra, 1994, Schmitz-Hoerner & Weissenböck, 2003), so this may most likely be the case in *M. polymorpha*. The importance of specific DNA repair genes up-regulated in *M. polymorpha* is discussed later in this chapter.

Heat shock proteins are involved in the stress response of plants and consist of molecular chaperones which help with correct folding and assembly of proteins (Treweek *et al.*, 2015). They have previously been found to be important in the response to UV-B (Murakami *et al.*, 2004b, Swindell *et al.*, 2007, Trautinger *et al.*, 1995). We saw higher up-regulation in high fluence treated plants at 1 day as compared to low fluence 1 day and high fluence 4 hour

time points. This is likely due to the higher stress exerted on high fluence plants and the increased need for heat shock proteins in order to reduce misfolding of proteins. A recent study also postulated that HY5 also has a role in mediating the up-regulation of genes for the unfolded protein response (UPR) (Nawkar *et al.*, 2017). Under normal light conditions HY5 bound a promoter element of UPR genes and kept expression at basal levels. However under stress conditions competition with a basic leucine zipper 28 (bZIP28) which bound the same element, together with degradation of HY5, increased expression of UPR genes (Nawkar et al., 2017). This finding shows the complicated cross talk that results in a combination of light signals mediated through HY5. HY5 may therefore be important in both the acclimation response through the UVR8 pathway but also the indirect stress response through its degradation and the up-regulation of UPR genes.

Chlorophyll changes are common in UV-B treated plants so a group of genes relating to the CAB proteins and those from chlorophyll biosynthesis were also analysed. CAB proteins were also seen to be highly expressed in response to high UV-B at 4 hours (Table 5.1). Group analysis showed that these genes were highly up-regulated early on in the UV-B response at 4 hours of high fluence and were down-regulated under both high and low 1 day treatments. The need for CAB proteins under UV-B may be required in order to help stabilise the light harvesting apparatus to keep chlorophyll functioning, a function such as provided by the CAB ELIP1 as previously discussed. Under high and low fluence 1 day samples no additional light is provided and plants have recovered after a dark period so CAB expression may be required less and hence we see the largely down-regulated trend at these time points. Chlorophyll biosynthesis genes were also looked at as chlorophyll levels may change under UV-B, although we did not see a change in *M. polymorpha*. Chlorophyll synthesis genes were both up and down in high 4 hour and low 1 day treatments but were largely down-regulated in high fluence 1 day samples. While we saw no detectable difference in total chlorophyll levels it may be that the synthesis of new chlorophyll in high fluence directly after the irradiance dose is not required as plants may focus on damage repair mechanisms rather than the synthesis of new chlorophyll. Chlorophyll biosynthesis in high fluence plants may occur at later time points however to keep steady state chlorophyll levels. Chlorophyll measurements were also conducted on whole plants which would include all tissue layers. Chlorophyll changes that could result from gene expression changes may be limited to those

areas of the thallus that are directly irradiated by UV-B. As such whole plant analysis may not be the best method as it would include thallus tissue that is shaded from UV-B by overlapping thallus pieces. A better measurement of chlorophyll changes would have been to take areas of thallus that would have received UV-B at high levels and measure the chlorophyll changes in those areas.

A group of chitin binding proteins was also analysed in order to help understand the response through pathogen related pathways. The chitin binding proteins include pathogen response genes such as PR-1 which in Arabidopsis has been shown to be induced by UV-B in a ROS dependent manner (Green & Fluhr, 1995, Mackerness et al., 2001). Putative chitin binding proteins were up-regulated in both the low and high fluence 1 day time points yet highly down-regulated in the high fluence 4 hour sample. This is likely due to the time of sampling as 4 hours into the UV-B treatment, even in high fluence, little damage has likely occurred and therefore any chitin binding gene mediated response may be unlikely to occur. At 1 day in high and low fluence the plants have been treated and the response through chitin binding proteins may indicate that damage to the plant has occurred. Interestingly both high and low fluence treatments showed similar response through the chitin binding proteins at 1 day. We would expect that high fluence treatment may have induced the chitin binding proteins more highly than low fluence as the higher fluence level would induce more damage. Plants are grown from gemmae for 5 weeks in UV-B lacking environments before they are ever introduced to UV-B so the response between both high and low fluence plants at the first irradiation event may cause a hypersensitive response in both as plants lack any UV-B acclimation such as increased flavonoids. As such both may respond to the first irradiation event similarly while later events in low fluence plants are protected through the high production of flavones. Ethylene response may also help mediate the UV-B response by regulating defence related genes (Mackerness *et al.*, 1999, Mackerness, 2000). Analysis of a group of ethylene response genes showed up-regulation in all treatments which suggests defence genes that are part of the chitin binding group may not be regulated by this group. However an ethylene responsive factor was highly up-regulated in the high fluence 4 hour and 1 day time points suggesting that ethylene may still play an important role in the response of *M. polymorpha*.

### 5.3.4 Flavonoid regulator genes and biosynthetic gene changes in *M. polymorpha* under UV-B conditions

The regulation of key genes in the UV-B response is well documented and largely mediated through the UVR8 UV-B photoreceptor. To understand how *M. polymorpha* responds UV-B treatments were sampled and RNA sequenced. This revealed that large changes in gene regulation occur. The main regulators of the direct UVR8 mediated pathway were found and showed that *M. polymorpha* responds to UV-B in much the same way as other plants. This was seen in the core set of regulators, RUP1, COP1, HY5, and SPA1 which regulate flavonoid biosynthesis in response to UV-B (Jordan, 2017). Importantly under UV-B we saw the increase in COP1, RUP1 and HY5 while SPA1 was relatively unchanged (Fig. 5.16, Table 5.4). Correspondingly under direct UV-B at 4 hours high fluence we also see the flavonoid biosynthetic genes up-regulated. In particular genes for PAL, CHS, CHI, FNS and FNR were found to be up-regulated (Fig. 5.17). Together we can see that under UV-B *M. polymorpha* responds through the UVR8 pathway and that this in turn regulates the flavonoid pathway to produce flavonoids.

While we found a HY5 gene that was up-regulated the downstream interaction of HY5 with other regulators such as the MYB genes is an important regulator of flavonoid synthesis in higher plants. MYB candidates were found of which a MYB14 gene showed up-regulation in low and high fluence at 1 day but was not highly up-regulated at 4 hours under UV-B (Table 5.4). This MYB was subsequently found to regulate riccionidinA in *M. polymorpha* (Albert and Davies Unpublished). The participation of this MYB14 may be largely involved in producing riccionidinA after UV-B events and could help in the stress response after UV-B irradiance. The effect of UV-B on over-expression and knockout lines of MYB14 is described in Chapter 6. It is likely that other unknown MYB transcription factors function to mediate the UV-B response in *M. polymorpha*.

A short under blue light (SUB1) homologue was also found that was up-regulated after the UV-B treatment in both the high and low 1 day samples. SUB1 has been proposed to negatively regulate HY5 during cryptochrome and phytochrome signalling (Guo *et al.*, 2001). Its up-regulation at 1 day in both high and low fluence may be related to this. The 1 day

sampling was early in the photoperiod for that day and the up-regulation may be due to expression during the dark period to negatively regulate HY5 in the dark. However the expression of HY5 has also been related to repair mechanisms through chaperone UPR genes to prevent misfolding under stress, so an alternate mode of action may be to reduce HY5 in order to up-regulate these UPR genes, such as the heat shock proteins we see highly up-regulated in the 1 day samples. Such regulation may modulate a need for the stress response after UV-B damage. Correspondingly we see that HY5 expression is high in the high fluence 4 hours time point while SUB1 expression is low. At 1 day, expression of HY5 has dropped while SUB1 has increased in expression, while COP1 which degrades HY5 in the dark is not up-regulated giving further evidence to SUB1's negative regulation of HY5 independent of COP1 (Guo et al., 2001). As HY5 defines a point of cross talk between light and UV-B signalling this regulation by SUB1 may be important for the balance of responses.

A basic helix loop helix (bHLH) gene encoding a homologue of GLABRA3 (GL3) was also found to be up-regulated under the 1 day time points in both high and low fluence. GL3 has been shown to be important for anthocyanin, trichrome and root hair production in Arabidopsis (Ramsay *et al.*, 2003, Koes *et al.*, 2005, Zhang *et al.*, 2003). The expression of this gene in *M. polymorpha* is down-regulated in high fluence 4 hour samples but highly up-regulated in 1 day samples suggesting it is not directly controlled in a UV-B dependent manner. It may however relate to the indirect response through a UV-B independent manner in order to increase the expression of flavonoids after UV-B events. Similarly a WRKY interacting protein was found to be highly expressed not only under UV-B at 4 hours of high fluence but also at high and low 1 day time points (Table 5.4). WRKY family transcription factors are involved in the response to both biotic and abiotic stress and also in flavonoid production and transport (Amato *et al.*, 2016, Rushton *et al.*, 2010, Eulgem & Somssich, 2007). The function of this WRKY interacting protein is speculative and would require functional analysis to determine its role in UV-B acclimation. However, its high expression during UV-B irradiance and also after irradiance at 1 day suggests it has a pivotal role.

An ERF is also highly up-regulated in both the high fluence 4 hour and 1 day time points. Ethylene is an important signalling molecule in plants and has been shown to be required for UV-B defence in Arabidopsis (Mackerness et al., 2001). The up-regulation of an ERF in *M.*

*polymorpha* under UV-B treatment suggests that ethylene response may also be important in UV-B signalling. How ethylene mediates plant defence in *M. polymorpha* is unknown but it may act similarly to Arabidopsis by increasing PR genes, which may include some of the putative chitin binding genes we see up-regulated in response to UV-B (Fig. 5.13).

While we are able to determine that the regulators and biosynthetic genes are up-regulated in response to UV-B under direct irradiance the expression of those genes under later time points was unknown. This was particularly interesting as we see that both high and low fluence UV-B induced flavonoid production yet only low fluence treatment has consecutive days of UV-B irradiance. Therefore the irradiance at day 1 in high fluence treatment is able to induce transcriptional changes that allow for the production of flavonoids well past the initial UV-B treatment with the highest accumulation at 4 days, 3 days after the UV-B treatment. We analysed the CHS gene for its expression under both low and high UV-B over a 4 day time period (Fig. 5.18). Irradiation of UV-B resulted in high levels of transcript abundance with increases starting at 4 hours and the highest difference observed at 12 hours of treatment. By 15 hours the low fluence treatment had reduced while high fluence transcripts were still higher than the control. However at 28, 76 and 100 hours the observable difference between all treatments was not significant. Sampling at these later time points were 4 hours into the UV-B treatment for low fluence and the corresponding time for high and control plants yet without UV-B. This time point is early in the treatment on these days and may not have provided enough time for transcripts to increase and thus we see no observable difference. Sampling at the end of the treatment time for these days would have been much more informative, i.e. 36, 84 and 108 hours. This would have corresponded to the 12 hour time point which may relate to the highest transcript abundance. It may also be that as transcripts increase and protein levels increase, additional transcription for protein is not required and some form of negative regulation restricts further transcription as not to over produce flavonoid compounds. This is seen in experiments with Arabidopsis were flavonoid levels were seen to accumulate to maximum amounts after the peak in mRNA abundance of key flavonoid biosynthetic genes (Kubasek *et al.*, 1992). Interestingly the peak in mRNA abundance of biosynthetic genes was followed 3 days later by the peak in flavonoid amounts which matched the difference we see also with mRNA peaking at 1 day and flavonoids peaking at 4 days. This peak in mRNA followed by a lag in flavonoid accumulation suggests that enzyme production on day 1 of UV-B treatment

allows for the production of flavonoids on corresponding days even without addition up-regulation of the biosynthetic genes. As such the initial up-regulation of the flavonoid genes at day 1 in high fluence plants is enough to result in the accumulation we see in flavones at 4 days. Such response may protect *M. polymorpha* by increasing flavones even in response to short bursts of UV-B.

### 5.3.4 Summary

This chapter illustrates the large changes that occur in the event of UV-B irradiance on *M. polymorpha*. *M. polymorpha* responds with induction of the flavonoid pathway mediated through UVR8. Key regulators such as HY5 may control the response through production of flavonoids. However ROS play a large role in the response to UV-B which may be enhanced in high fluence treatments. A summary of response relating to a number of gene changes seen is shown in Figure 5.19. UV-B may be sensed through the UVR8 photoreceptor which activates signalling through regulators such as RUP1, COP1 and HY5. Accumulation of HY5 in conjunction with other transcriptional activators may then activate the flavonoid biosynthetic genes for the production of flavonoids and in particular the flavones. HY5 accumulation may also result in the activation of ELIP genes for protection of the photosynthetic machinery. In the stress response, independent of UV-B, SUB1 accumulation may degrade HY5 and up-regulate UPR genes for the maintenance of protein folding. The synthesis of flavones in *M. polymorpha* may then function in two ways through UV-B screening by localisation to epidermal layers and also through the reduction of ROS. Flavones produced in the cell may directly scavenge ROS while polyphenol and copper oxidases may help oxidise flavonoids and produce brown pigments in the cell with the potential to also scavenge ROS. UV-B may indirectly act through the accumulation of ROS in the chloroplasts and this may be reduced through the activation of copper chaperones and peroxidases. CAB and ELIP up-regulation may also help stabilise the photosynthetic machinery. Generalised stress response may occur through the misfolding of proteins which may also be indicative of increased ROS burden and as such *M. polymorpha* responds through the up-regulation of heat shock proteins and chaperones. Ethylene sensing through an ethylene responsive factor may also indicate that ethylene plays a role through chitin

binding and PR genes. DNA which absorbs UV-B directly and also is susceptible to ROS products is repaired through the up-regulation of DNA glycosylases and photolyases.

Overall we see large changes through not only flavonoid production but ROS production and stress response in order to maintain cellular function. Such machinery, particularly the induction of flavonoids in *M. polymorpha*, would likely have resulted in protection against UV-B in the early land plants of which *M. polymorpha* is the closest extant relative.

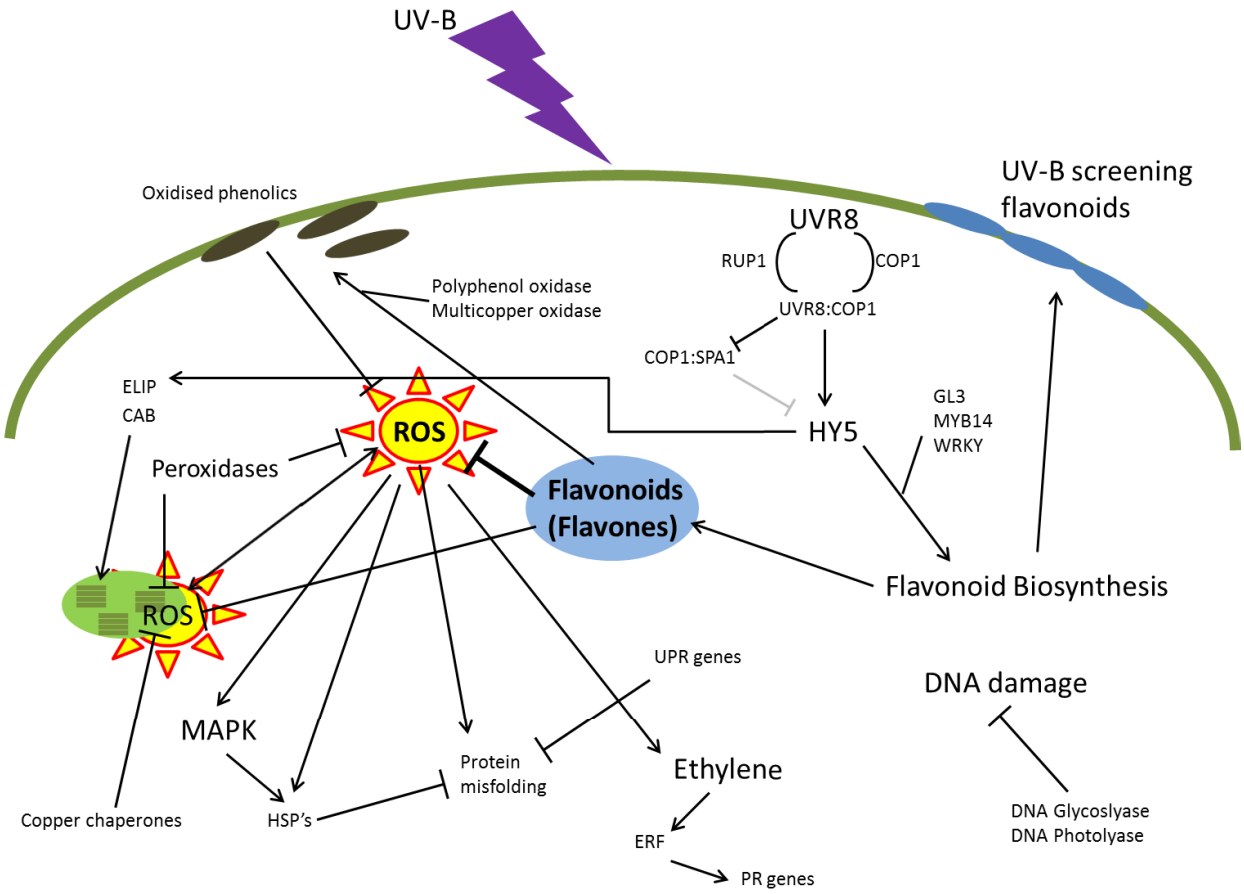



Figure 5.19: A summary of the response to UV-B in *M. polymorpha.*

# Chapter 6: Mutation analysis of flavonoid response genes

## 6.1 Introduction

After the discovery of UV-B responsive genes in *M. polymorpha* using RNA sequencing, mutants for key transcriptional activators and flavonoid pathway genes were created (Albert and Davies Unpublished). The most interesting of which were mutants of chalcone isomerase (CHI), chalcone isomerase-like (CHI-L), repressor of UV-B photomorphogenesis (RUP1), elongated hypocotyl 5 (HY5) and a MYB type transcription factor (MYB14). *M. polymorpha* was previously shown to respond to UV-B at both high and low fluence with the production of flavonoid compounds (Chapter 4). Therefore the disruption of the key biosynthetic or regulatory genes was important to understand the contribution of such flavonoids to the protection of *M. polymorpha* from UV-B.

CHI catalyses the step from chalcones to flavonones and has previously been used to modify the flavonoid profile in Arabidopsis. A mutant in Arabidopsis was highly sensitive to UV-B irradiance (Li *et al.*, 1993b) while overexpression of a petunia CHI gene in tomato resulted in a large increase in flavonol levels (Muir *et al.*, 2001). Similarly CHI-L genes are thought to be involved in the step converting chalcones to flavonones but they may not have enzymatic activity (Morita *et al.*, 2014). Knockdown mutations of CHI-L genes in petunia (*Petunia hybrida*) and torenia (*Torenia hybrida*) resulted in significantly lower amounts of flavonols, flavones and anthocyanins, suggesting an enhancer function of CHI-L genes (Morita et al., 2014). As flavones are enhanced in *M. polymorpha* after UV-B irradiation the disruption of CHI and CHI-L genes would be important in understanding the role of flavones in UV-B protection.

To allow plants to respond to UV-B wavelengths, signal transduction likely also occurs through the direct UVR8 mediated pathway. A key regulator of this pathway is the gene encoding repressor of UV-B photomorphogenesis (RUP1). This gene was highly up regulated under UV-B conditions in RNA-seq data and is therefore a key target to knock out. RUP1 is required to recycle UVR8 from its active monomer back to its inactive dimer state by disrupting COP1 interaction and thus halting signal transduction (Heijde & Ulm, 2013). Mutants in Arabidopsis of RUP1 and RUP2 result in an enhanced UV-B response and elevated tolerance to UV-B while overexpression of RUP1/2 results in hypersensitivity to UV-B

(Gruber et al., 2010). HY5 is a bZIP transcription factor that acts in a light and UV-B dependent manner for the activation of a wide range of genes including those involved in flavonoid biosynthesis (Ulm *et al.*, 2004). The mutant of HY5 in Arabidopsis was highly sensitive to UV-B irradiation (Brown & Jenkins, 2008). Similar to HY5 other transcription factors interact with downstream genes to enhance the UV-B response. Many MYB transcription factors have been identified and the transcription factor MYB14 was identified in *M. polymorpha* after showing high up-regulation at 1 day after both high and low fluence treatments.

*M. polymorpha* WT and mutant lines were tested to determine the production of flavonoid compounds and the effect that this may have on the acclimation response to UV-B.

## 6.2 Results

### 6.2.1 UV-B response mutants of *M. polymorpha* have significant changes in flavone production

To determine if mutation or overexpression of key genes in the UV-B pathway resulted in changes to flavone accumulation, plant lines were subject to the different UV-B treatments, control, high and low UV-B, and were analysed by UPLC for flavone amounts. Apigenin-based flavones were those comprising of apigenin 7 mono-*0*-glucuronide. Luteolin-based flavones were those of the combined amounts of Luteolin 7,4' di-*0*-glucuronide, Luteolin-di 7,3'di-*0*-glucuronide and Luteolin 4'-mono-*0*- glucuronide.

Total flavone amounts were found to increase under low fluence conditions with a smaller but still significant increase under high fluence conditions. As compared to WT under control conditions *chi* and *chi-l* mutants had significantly lower flavone levels with the *chi* mutant producing no detectable flavones. The *rup1* mutant and the 35s:MYB14 over expressing line

both had significantly higher flavone levels while the *hy5* and *myb14* mutants showed no significant change from WT under control conditions (UV-) (Fig. 6.1).

Total flavones under low UV-B fluence conditions, as compared to WT, showed significantly lower flavone amounts in the *chi*, *chi-l* and *hy5* mutants. Higher amounts of total flavones were present in the *rup1* mutant and 35s:MYB14 over expression line, while the *myb14* mutant showed no significant change in flavone level (Fig. 6.1). The *hy5* mutant did not follow the trend of normal flavone response under low fluence conditions with no increase seen compared to WT plants (Fig. 6.1). This was also illustrated in the fold change of total flavones as compared to the UV- control (Fig. 6.2). WT plants showed a 2 fold increase under low fluence conditions and a 1.25 fold increase under high fluence conditions. This trend was followed in all other plant lines except for the *hy5* mutant which showed no response in low fluence conditions (Fig. 6.2).

Under high fluence conditions *chi*, *chi-l* and *hy5* mutants had lower flavone levels while the *rup1* mutant and 35s:MYB14 lines had higher levels of flavones. *myb14* mutants showed no significant difference from WT (Fig.6.1).

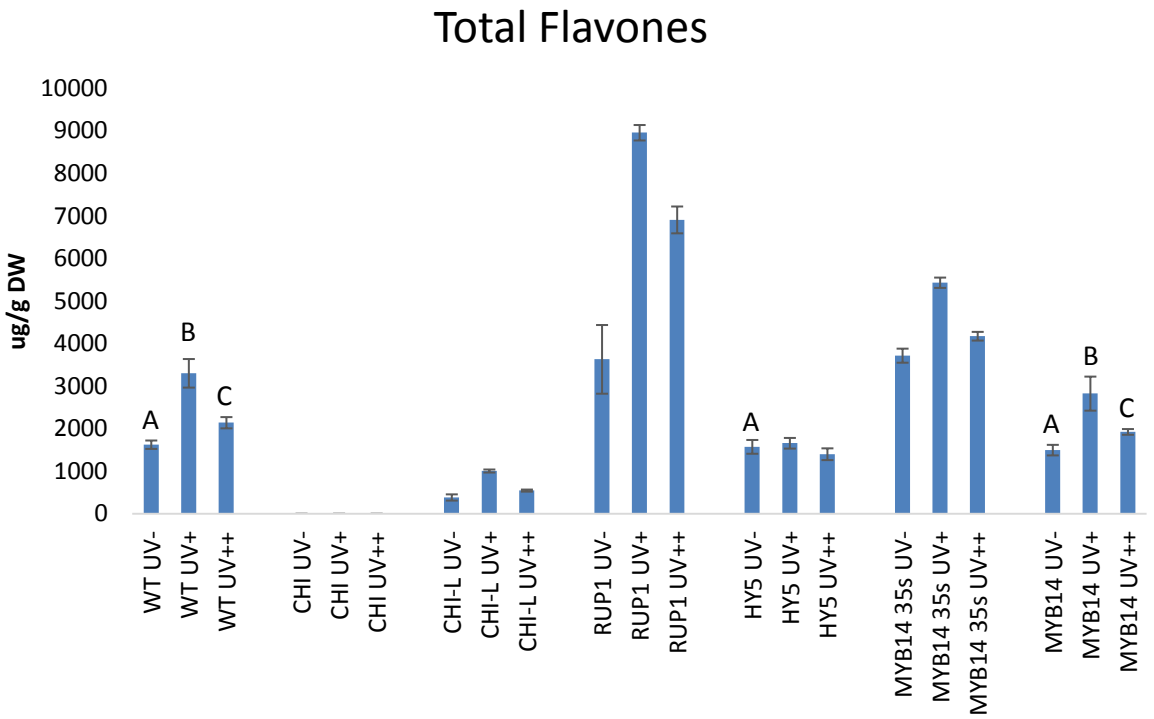



Figure 6.1: Total flavone amounts of mutants for genes involved in UV-B response of *M. polymorpha.* Total flavones are measured in µg/g DW. Control (UV-), low fluence (UV+) and high fluence (UV++) responses are shown. Amounts in mutants are compared to the WT control at 95% confidence interval. Letters indicate non-significant difference from WT control response within each UV-B treatment group ($p<0.05$). Error bars = Standard error (n=3).

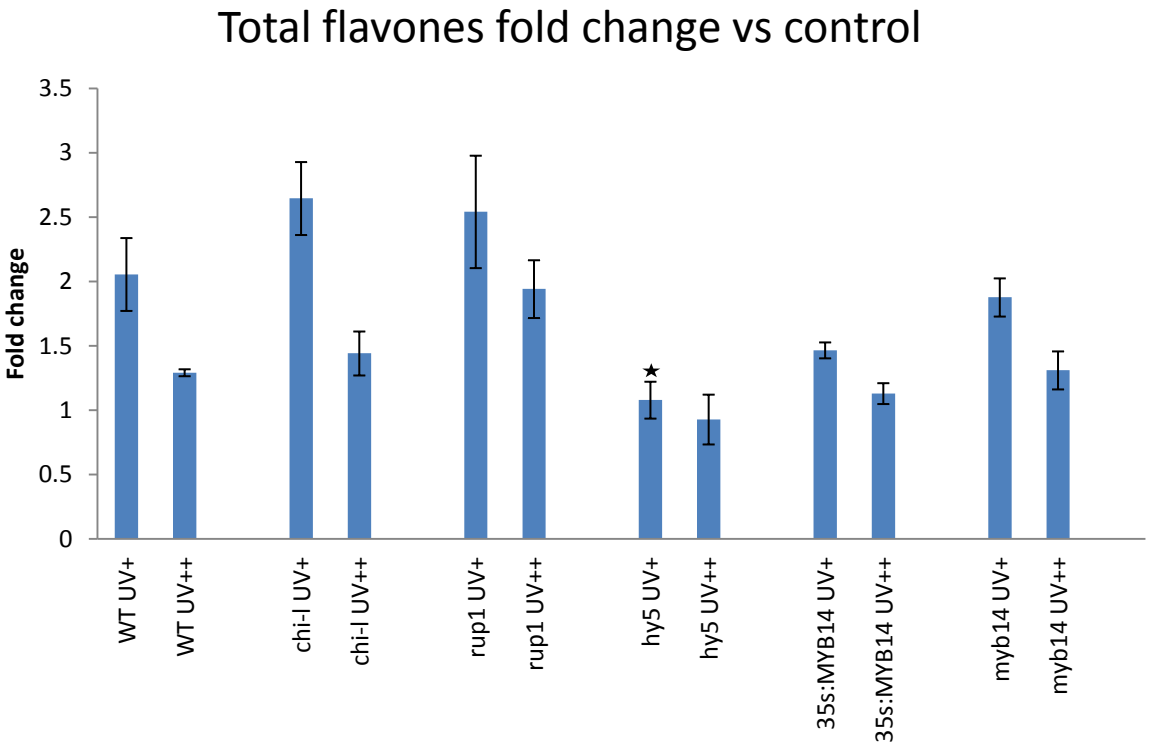



Figure 6.2: Total flavone amounts fold change vs control. The fold change of total flavones as compared to the non-UV-B control for low (UV+ and high (UV++) fluence treatments. WT was used as a control group to determine any significant differences in fold change response (*$P$<0.05). Error bars = Standard error (n=3).

While total flavone levels were seen to change within *M. polymorpha* the makeup of flavones being apigenin or luteolin-based may also be important. For each line the contribution of apigenin and luteolin-based flavones was also measured. In WT plants apigenin-based flavones followed the same trend as total flavones with increases under low fluence and high fluence treatments (Fig. 6.3). The *chi* mutant contained no apigenin-based flavones. The *chi-l* mutant responded similarly to WT but had significantly reduced levels of apigenin-based flavones under all treatment conditions. The *rup1* mutant had elevated levels of apigenin-based flavones under all treatment groups but followed the same trend with the largest increase seen under low fluence conditions. The *hy5* mutant showed no significant difference to WT in untreated plants but had significantly reduced levels of apigenin-based flavones in low and high fluence treatments. Apigenin-based flavones did not show any significant increase or decrease in the *hy5* mutant under the different treatments.

35s:MYB14 over expressing lines had significantly higher apigenin-based flavones in control and high fluence treatments but no significant difference from WT was seen under low fluence conditions. The *myb14* mutant showed no significant difference from WT under any treatment condition (Fig. 6.3).

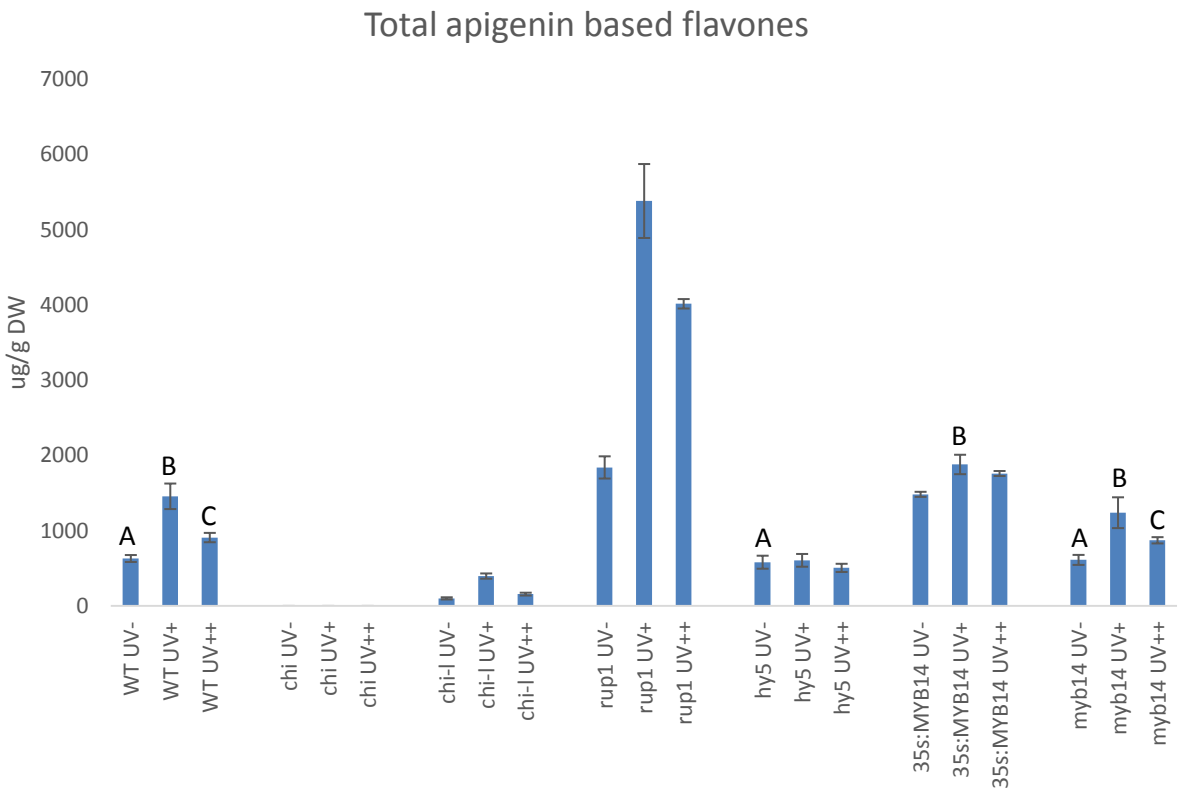



Figure 6.3: Total apigenin-based flavone levels of mutants involved in UV-B response of *M. polymorpha.* Totals are measured in µg/g DW. Control (UV-), low fluence (UV+) and high fluence (UV++) responses are shown. Amounts in mutant lines are compared to the WT control at 95% confidence interval. Common letters indicate non-significant difference from WT response within each UV-B treatment group ($p<0.05$). Error bars = Standard error (n=3).

In WT luteolin-based flavones also followed the same trend as total flavones with increases under low fluence and high fluence treatments (Fig. 6.4). The *chi* mutant contained no luteolin-based flavones. The *chi-l* mutant responded similarly to WT but had significantly reduced levels of luteolin-based flavones under all treatment conditions. The *rup1* mutant had elevated levels of luteolin-based flavones under all treatment groups but followed the same trend with the largest increase seen under low fluence conditions. The *hy5* mutant showed no significant difference to WT in untreated plants but had significantly reduced levels of luteolin-based flavones in low and high fluence treatments. Luteolin-based flavones

did not show any significant increase or decrease in the *hy5* mutant between treatments. 35s:MYB14 over expressing lines had significantly higher luteolin-based flavones under all treatment conditions as compared to WT. The *myb14* mutant showed no significant difference from WT under any treatment condition (Fig. 6.4).

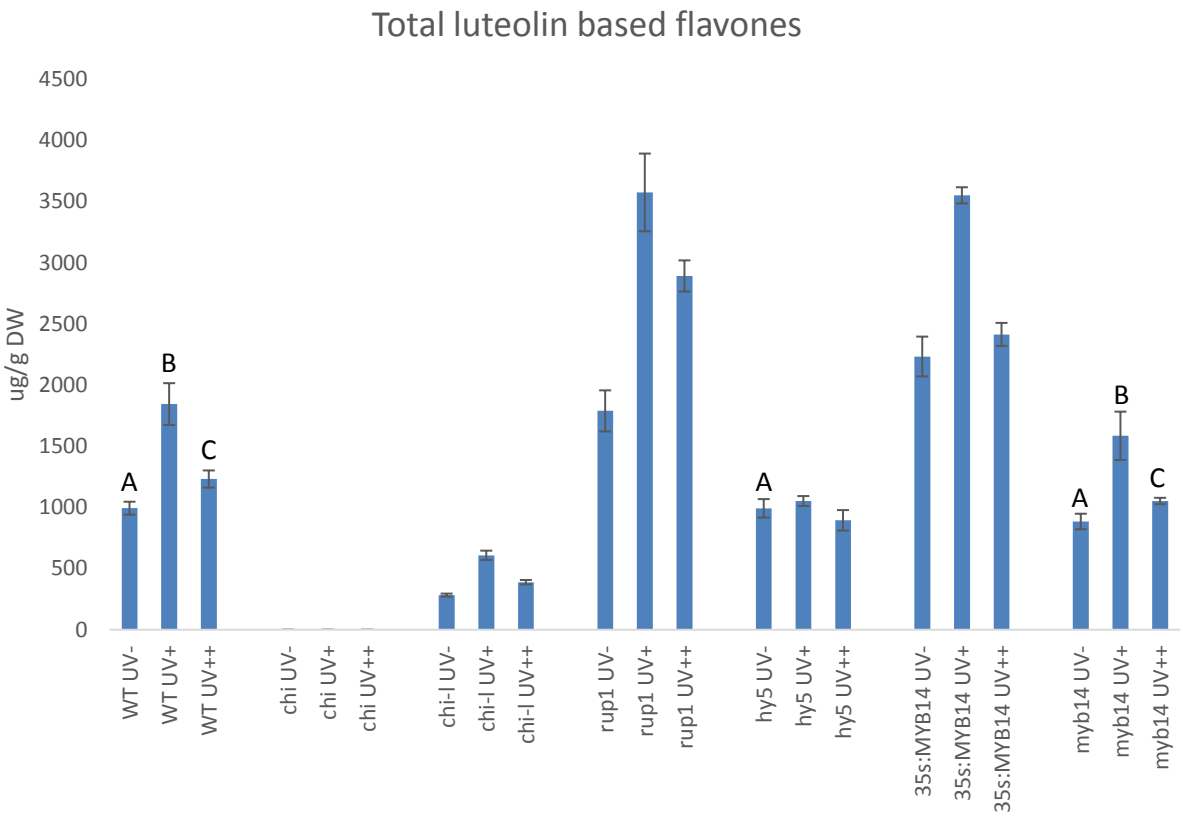



Figure 6.4: Total luteolin-based flavone levels of mutants involved in UV-B response of *M. polymorpha.* Totals are measured in µg/g DW. Control (UV-), low fluence (UV+) and high fluence (UV++) responses are shown. Amounts in mutant lines are compared to the WT control at 95% confidence interval. Common letters indicate non-significant difference from WT response within each UV-B treatment group ($p<0.05$). Error bars = Standard error (n=3).

The ratio of apigenin to luteolin-based flavones was determined for each line and any significant differences from WT determined (Fig. 6.5). The *chi-l* mutant had significantly reduced ratios of apigenin to luteolin under control and high fluence conditions but no significant difference was seen under the low fluence treatment. The *rup1* mutant had an elevated apigenin to luteolin ratio under all treatment conditions. The *hy5* mutant had no

significant difference in ratio under control treatments but significantly lower ratios under low and high fluence treatments. The ratio in *hy5* mutant plants did not change under any treatment group. 35s:MYB14 over expression plants showed no significant difference under control and high fluence conditions but a reduced ratio of apigenin to luteolin was seen under low fluence conditions. The *myb14* mutant showed no significant difference in the ratio of apigenin to luteolin-based flavones under any treatment (Fig. 6.5).

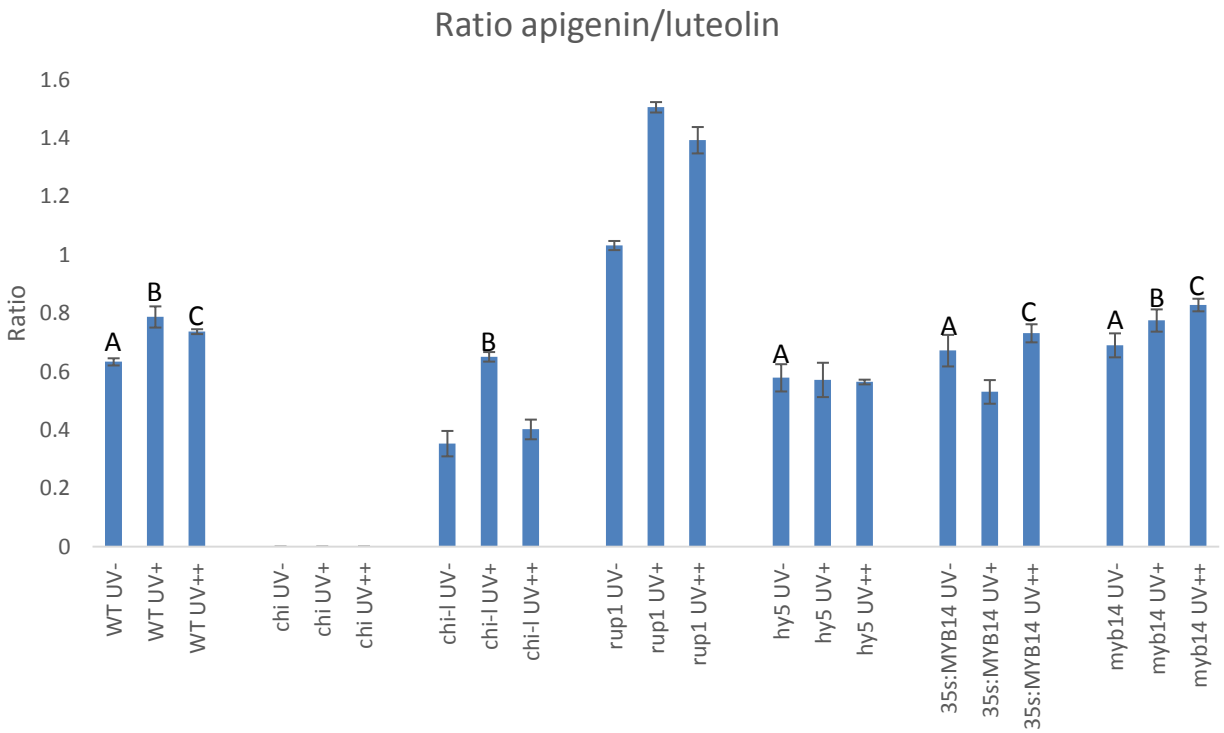



Figure 6.5: Ratio of apigenin to luteolin-based flavones in mutants involved in the UV-B response of *M. polymorpha*. Control (UV-), low fluence (UV+) and high fluence (UV++) ratios are shown. Amounts in mutant lines are compared to the WT control at 95% confidence interval. Common letters indicate non-significant difference from WT response within each UV-B treatment group ($p<0.05$). Error bars = Standard error (n=3).

### 6.2.2 Flavone levels corresponding to phenotypic difference in *M. polymorpha*

To determine the phenotypic effect of UV-B on each mutant and over expressing line, plants were photographed after treatment and the flavonoid content measured. Under control (UV-) conditions it could be seen that all plant lines grew well with no visible signs of stress such as bronzing, browning or increased surface reflectance (Fig. 6.6). Loss of flavones in the *chi* mutant could be seen under flavonoid specific DPBA staining as a lack of green fluorescence while the increased levels of flavones in the *rup1* mutant and 35s:MYB14 over expresser could be seen by high levels of fluorescence throughout the surface of the thallus tissue. 35s:MYB14 over expression plants are a red colour due to the increased levels of riccionidinA (Albert and Davies Unpublished).

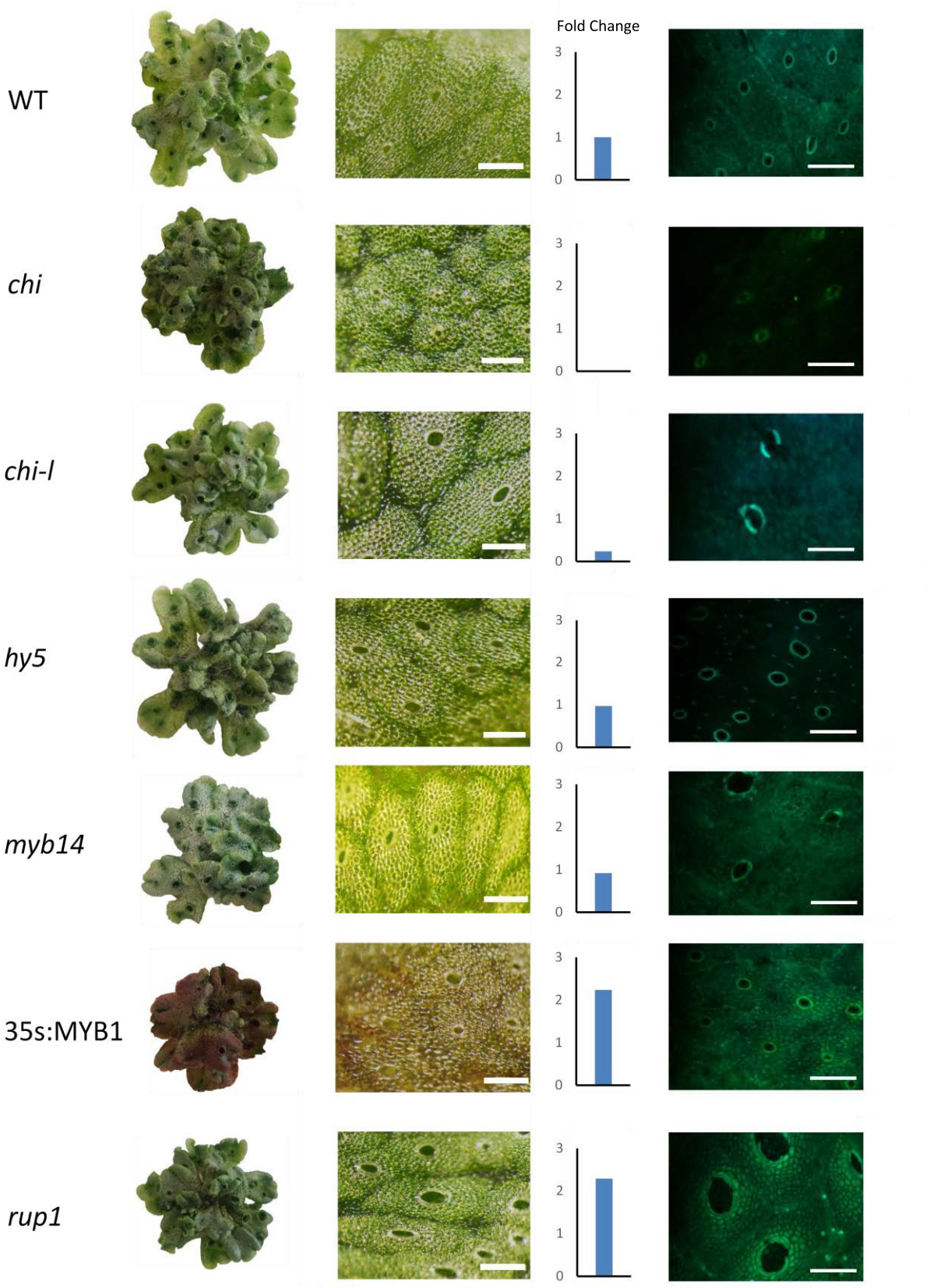



Figure 6.6: Phenotypes of UV-B response mutants under control conditions. For each line whole plant photos are shown with dissecting micrographs at 10x magnification of thallus tissue (Scale bars = 20 μm). The fold change in total flavone levels under control conditions as compared to WT is shown. Fluorescent micrographs showing localisation of flavonoid compounds in the thallus is also shown (Scale bars = 100 μm).

Low fluence treatment resulted in no detectable damage to WT with normal surface structure and greening (Fig. 6.7). The *chi* mutant lacked flavones and distinct browning of the thallus surface could be seen in whole plants. Dissecting micrograph images showed loss of greening. This same phenotype could be seen in the *hy5* mutant but not as severe, while *chi-l* mutants also displayed browning of the thallus but under dissecting micrograph the thallus surface damage was not as severe as *chi* or *hy5* mutants (Fig. 6.7). All three *chi*, *chi-l* and *hy5* mutants had reduced flavones under low fluence treatment as compared to WT. *myb14* mutants showed no difference from that of WT in whole plant or thallus surface phenotype and the flavone response was the same as WT. 35s: MYB14 over expression and the *rup1* mutants showed no signs of damage. A darkening of tissue was observed in *rup1* mutants (Fig. 6.7), and isolated areas of increased riccionidinA could also be seen in *rup1* mutants (Fig.6.9).

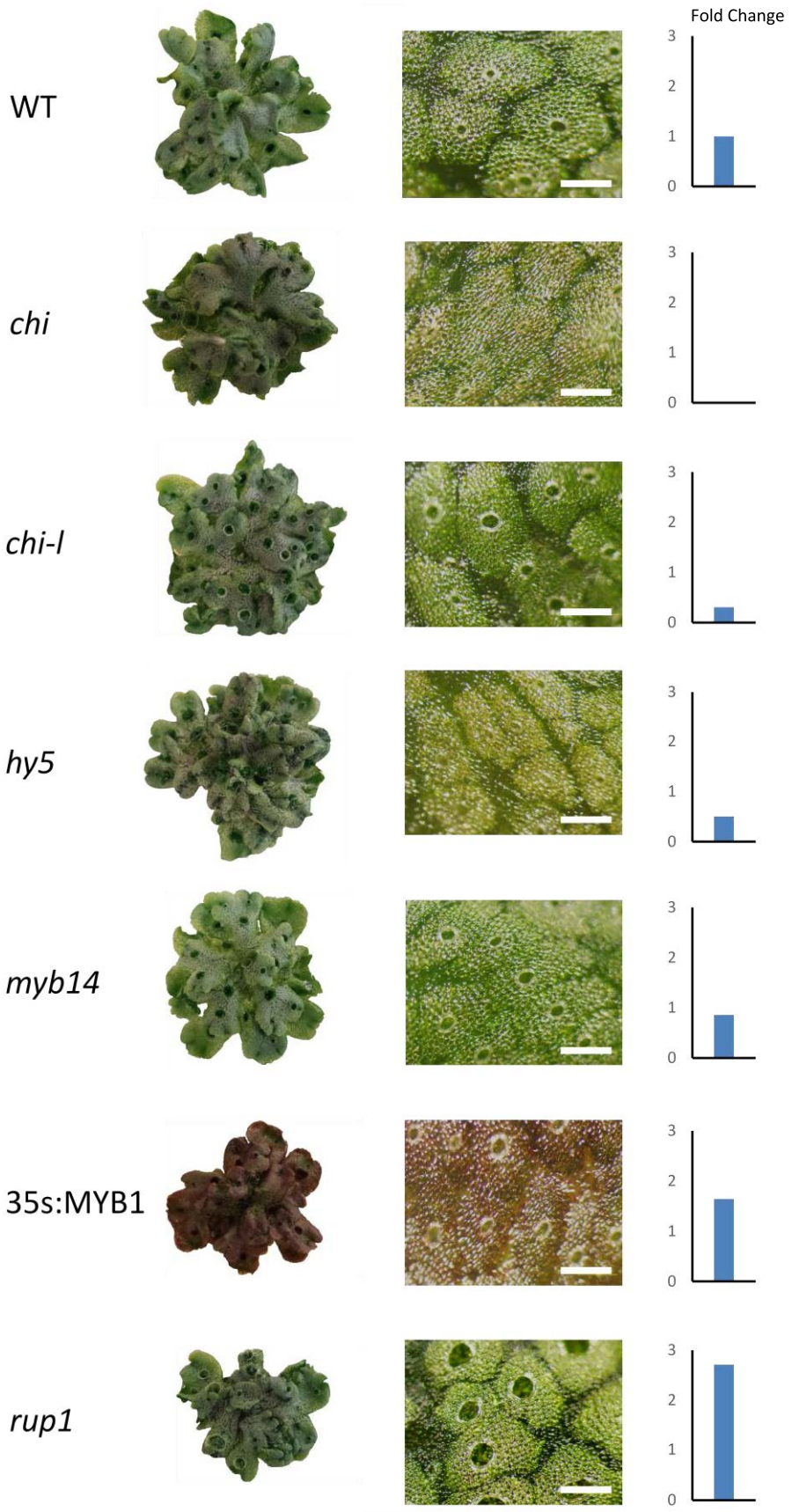



Figure 6.7: Phenotypes of UV-B response mutants under low fluence conditions. For each line whole plant photos are shown with dissecting micrographs at 10x magnification of thallus tissue (Scale bars = 20 μm). The fold change in total flavone levels under low fluence conditions as compared to WT is shown.

High fluence treatment caused damage to WT plants with surface bronzing and a loss of greening (Fig. 6.8). *chi*, *chi-l* and *hy5* plants showed high levels of surface bronzing in whole plants and also a loss of greening as seen under dissecting microscope. Flavones were reduced under high fluence in these mutants as compared to WT (Fig. 6.8). *myb14* mutants showed similar damage to WT with less whole plant damage seen but similar thallus damage seen in the loss of greening and disruption of surface structure reflectance. 35s:MYB14 over expression plants did not display any bronzing phenotype in whole plant analysis or in thallus surface analysis. The thallus was much redder under high fluence treatments in 35s:MYB14 over expressing plants than in control or low fluence treatments. Flavone amounts in these plants were elevated as compared to WT. *rup1* mutants showed no bronzing of the surface and no loss of greening (Fig. 6.8). Dark areas in whole plant analysis could be seen but on closer inspection this was not due to damage but an increased level of riccionidinA production (Fig. 6.9). *rup1* mutants exhibited no change under high fluence conditions and were tolerant to low and high UV-B treatments. The *rup1* mutants had elevated amounts of flavones under high fluence as compared to WT (Fig. 6.8).

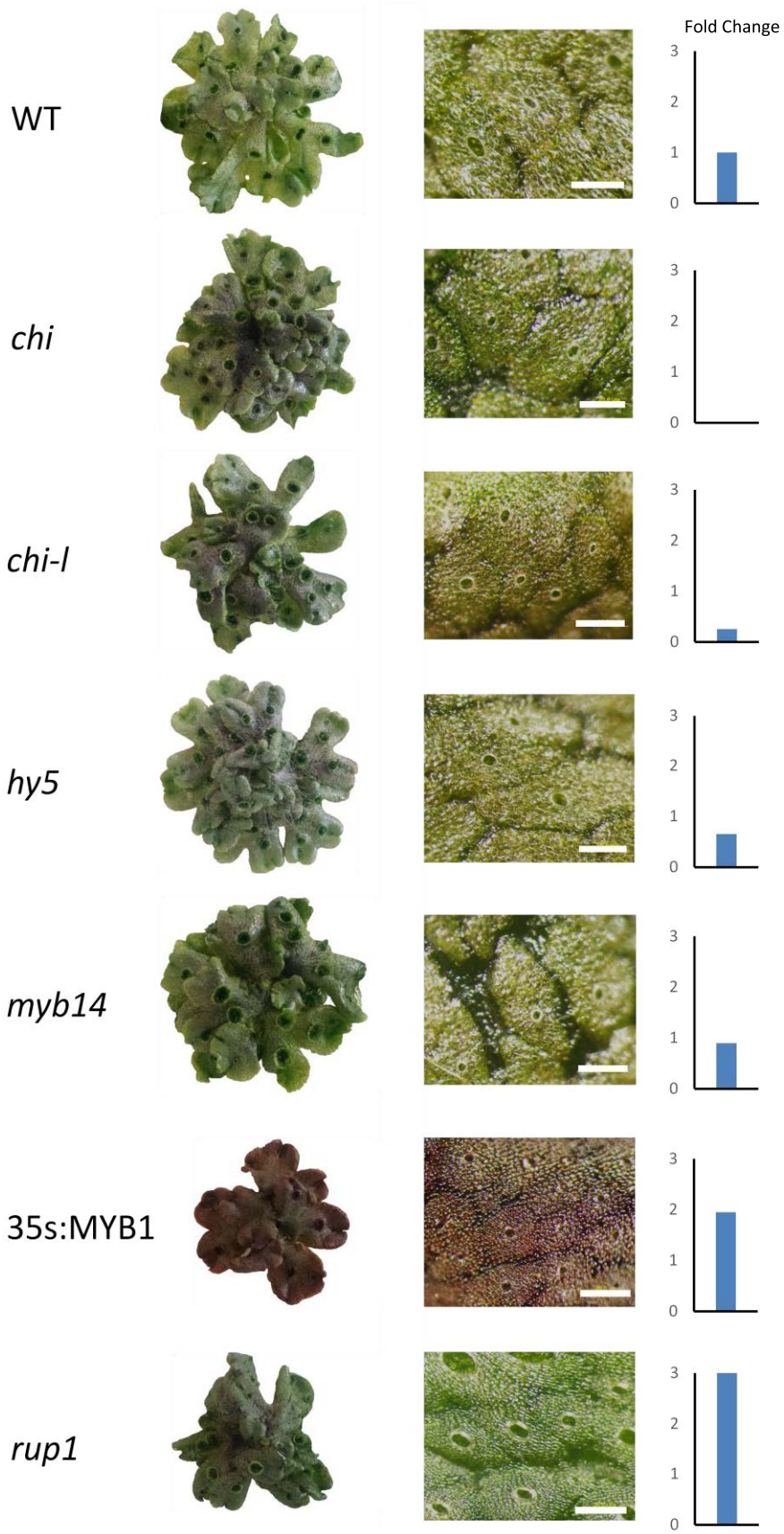



Figure 6.8: Phenotypes of UV-B response mutants under high fluence conditions. For each line whole plant photos are shown with dissecting micrographs at 10x magnification of thallus tissue (Scale bars = 20 μm). The fold change in total flavone levels under high fluence conditions as compared to WT is shown.


Figure 6.9: Isolated production of riccionidinA in RUP1 mutants. Dissecting micrographs of *rup1* mutants under low (UV+) and high (UV++) treatments. Areas of riccionidinA production (arrows) occur under both treatments but production is not seen under control (UV-) treatment.

To determine the long term ability of plants to acclimatise to UV-B, *M. polymorpha* lines were grown under low fluence conditions for an extended period of 4 weeks (Fig. 6.10). WT plants showed a slight decrease in plant growth and a darkening of thallus tissue. *rup1* plants showed severe stunting under UV-B conditions as compared to control (UV-) conditions. *hy5* mutants showed a stunted phenotype which although not as severe as RUP1 mutants, plants were more stunted than WT plants. The *myb14* and *chi-l* mutants showed a slight reduction in growth as compared to that of WT treated plants. 35s:MYB14 over-expression lines showed similar reduced growth and an increase in red colour of the thallus tissue. *chi* mutants which had lost the ability to produce flavones were severely stunted and had abnormal thallus growth. Thallus tissue grew tightly and was wavy in appearance (Fig. 6.10).

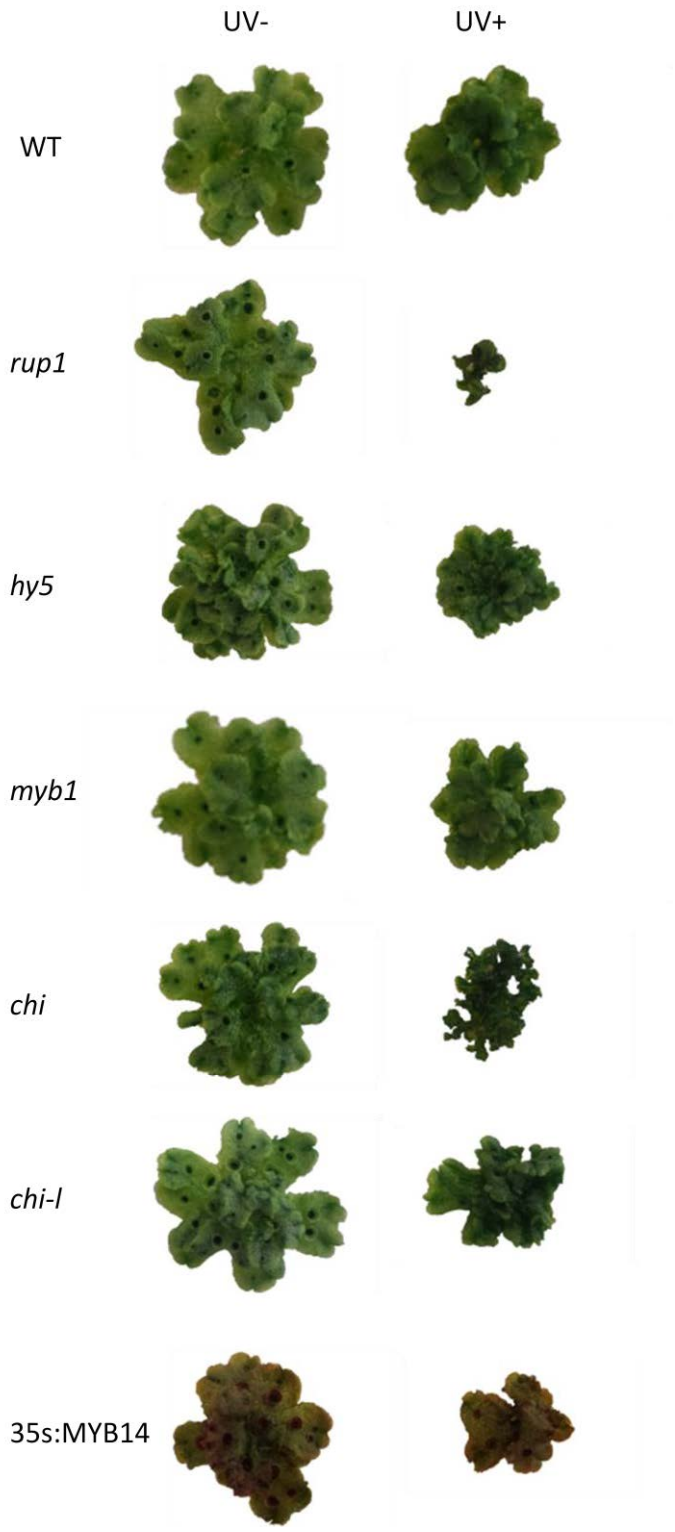



Figure 6.10: Growth response of UV-B response mutants under acclimatisation conditions. Plants were grown under control (UV-) and low fluence (UV+) UV-B and photographed after 5 weeks.

Plants respond to UV-B through the production of ROS which can act as both a signalling compound at low amounts, or damaging stressor at high amounts. *M. polymorpha* WT had previously been shown to accumulate high amounts of ROS in the chloroplasts in response to high fluence UV-B, which may act in the stress response and damage seen in these plants. To determine how ROS might function in plants that have modified production of flavones WT, *chi*, *rup1* and UVR8 OE plants were subject to high fluence UV-B and stained for the localisation of ROS at 12 hours post treatment (Fig. 6.11). ROS accumulation was seen in the chloroplasts of WT plants but this localised to limited areas that were most directly exposed to UV-B. *chi* mutants showed very high levels of ROS accumulation within the chloroplasts and this was largely spread over the entire thallus tissue. *chi* plants do not produce flavones, which indicates flavones may function in ROS scavenging. Comparatively, *rup1* mutant plants produce very high amounts of flavones and we observed no accumulation of ROS within the chloroplasts in thallus tissue (Fig. 6.11). Similarly the UVR8 overexpressor was found to produce high levels of flavones (Nick Albert, personal communication, 2017) and we observed no chloroplast ROS accumulation in thallus tissue of these plants. Overall plants that had reduced flavones had increased ROS accumulations while those that had high levels of flavones had no ROS accumulation in thallus tissues.

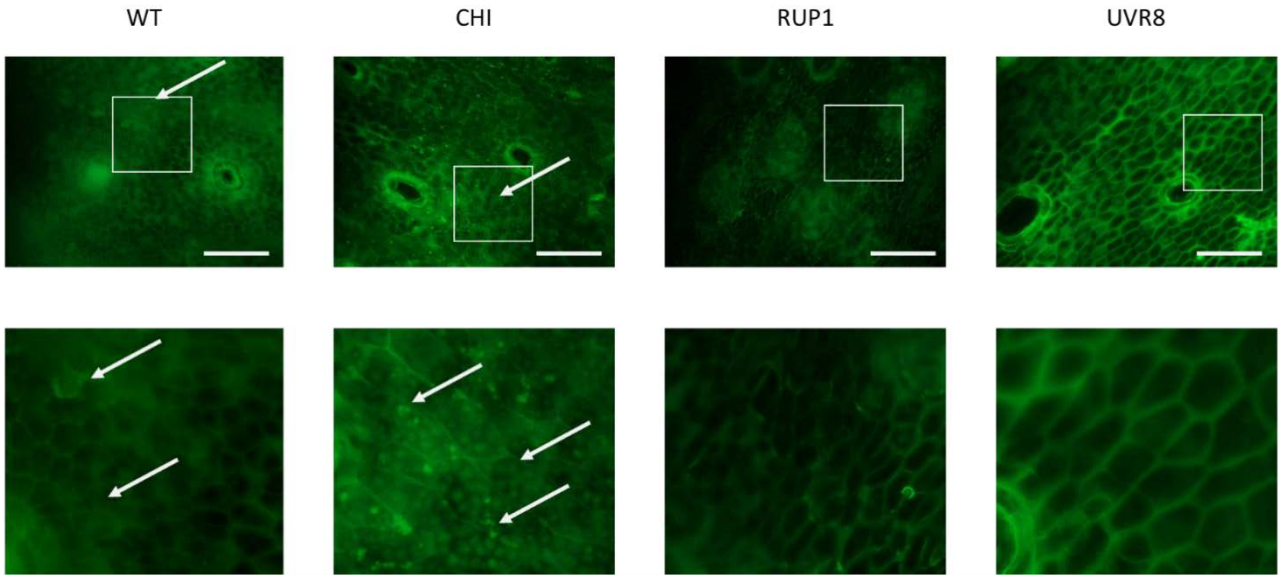



Figure 6.11: ROS accumulation in response to high fluence UV-B in *M. polymorpha* plants with modified flavone production. Thallus tissue stained with DCFA which fluoresces upon oxidation by ROS. Arrows indicate ROS accumulation in chloroplasts. White boxes indicate areas of magnified images. Scales bars = 100 µm.

## 6.3 Discussion

### 6.3.1 UV-B response mutants of *M. polymorpha* have significant changes in flavone accumulation

WT *M. polymorpha* responds to UV-B through the production of flavonoid compounds under high and low fluence and this induction is likely largely mediated through the UVR8 pathway. Several candidate genes for regulators of the UVR8 and biosynthetic pathway were found by RNA-sequencing, and mutant and over-expression lines were analysed to determine how they would respond to UV-B.

CHI

Flavones were absent from *chi* mutants which is understandable as CHI is required for the conversion of chalcones to naringenin and subsequent flavone production, and the CHI candidate in *M. polymorpha* was identified as a single copy gene. The reduction in flavones was easily seen when the thallus tissue was stained for flavonoid compounds (Fig. 6.6). This reduction also resulted in a hypersensitive response to UV-B at both high and low fluences indicating the importance of flavones in UV-B protection. Plants showed increased browning of the thallus indicative of damage in both high and low fluence conditions as compared to the wild type. This increased browning may likely be due to the lack of UV-B screening ability as plants had very little flavonoid content in the epidermal layers. Such reduced UV-B screening capacity would result in very little protection against incident UV-B and so we see the increased thallus damage in *chi* mutants. When *chi* mutant plants were plated from gemmae and grown under constant low fluence UV-B a severe stunting phenotype was also observed (Fig. 6.10). This stunting was likely due to the loss of flavone production that is unable to ameliorate the effects of UV-B, as plants grown under the same conditions, but without additional UV-B, did not show any phenotypic difference. This indicates that the loss of flavones did not alter the growth characteristics under conditions lacking UV-B and stunting observed under added UV-B was due to UV-B and not a loss of other plant functions relating to flavones, such as auxin transport in growing tissue. The importance of flavones in *M. polymorpha* for the protection against UV-B must therefore be very important. This result

is similar to that seen in Arabidopsis with the loss of CHI function resulting in a hypersensitive response to UV-B due to decreased flavonoids (Li et al., 1993a).

CHI-L

Flavones were detected in the *chi-l* mutant which suggests *chi-l* is not essential for flavonoid production. *chi-l*L plants had very low levels of total flavones which suggests that *chi-l* has an enhancer activity similar to that previously described in higher plants (Morita et al., 2014). While overall the concentrations of total flavones were reduced in the *chi-l* mutant the response to UV-B as seen by the fold change of flavones under low and high fluence was not significantly different from WT. This suggests that although CHI-L is an enhancer of the flavonoid biosynthetic pathway it is not a regulator of the pathway mediated through UV-B. The ratio of apigenin to luteolin was significantly lower in the control and high fluence plants which suggest that *chi-l* mutation results in modification of the production of luteolin or apigenin-based flavones in these treatments. The low fluence treatment showed no difference in ratio to that of wild type suggesting that under low fluence conditions apigenin production is favoured, similar to that of the WT response. *chi-l* plants under UV-B treatments showed damage phenotypes in both low and high fluence treatments although low fluence, while still damaging, was less severe. The loss of CHI-L may decrease flavonoid production through the loss of its enhancer function and subsequently when plants are challenged with UV-B the lower flavone level is insufficient for full protection. However acclimation testing of *chi-l* mutants showed that they were able to grow under UV-B light, although they were slightly stunted. The reduced levels of flavones in these mutants may, although not high enough for full UV-B protection, still be enough to allow for normal growth function rather than the full stunting we see in *chi* mutants. Importantly the increased damage in plants that were treated at high and low fluence may be due to a lack of UV-B screening as little flavone content was observed in the epidermal layers. However plants grew relatively normally under long UV-B acclimation exposure suggesting that the limited amount of flavones present, while potentially not acting initially in a UV-B screening capacity, were able to allow for plant growth potentially through ROS scavenging activity or later localisation to epidermal layers. This may also explain the *hy5* phenotype with a similar reduction in flavone level.

HY5

HY5 is a major regulator of light responses and also the UV-B response. Knockouts were able to produce flavones in *M. polymorpha* yet flavone production was unresponsive to UV-B. Total flavones remained unchanged between control, low and high fluence treated plants. No differences in the ratio of apigenin to luteolin were seen from control plants also suggesting no conversion between apigenin or luteolin under high or low UV-B treatments. *hy5* mutants had the same levels of flavones as WT plants under conditions lacking UV-B suggesting that under conditions without UV-B other regulators are able to induce the flavonoid genes. However once UV-B is sensed by the plants, without HY5, they are unable to respond through the production of flavones suggesting HY5 is a major regulator of the UV-B response in *M. polymorpha*. Plants grown under control conditions and then submitted to low and high fluence conditions showed hypersensitive response with damage seen in whole plants under both conditions. This suggested that similar to the *chi-l* mutant, the presence of flavone compounds alone is not enough to give protection and in order to protect against UV-B damage plants must respond through an enhanced production of flavones to mitigate damage. However under constant UV-B acclimation plants were able to grow but in a stunted phenotype which suggests that the presence of flavones is enough to help protect plant function in long acclimation responses rather than sudden changes, such as the high fluence treatment procedure.

MYB14

A MYB transcription factor MYB14 was found that was up-regulated in response to both high and low UV-B (Chapter 5). This transcription factor was studied for the production of riccionidinA (Albert and Davies Unpublished). The mutant and over expressing lines were used in this study to determine how this MYB may affect flavone concentration in response to UV-B and subsequently any protective functions.

MYB14 over-expressor

The MYB14 overexpressor was seen to have high accumulation of the red pigment riccionidinA in whole plants and also an increased production of flavones. Irradiation with

UV-B resulted in increases in the flavone concentration and this response was similar to that of the WT response with no significant difference seen in the fold change of total flavones. This showed that although innate flavone concentrations were elevated, the response through the UV-B pathway was not enhanced by over-expression of MYB14, suggesting that MYB14 may act independently of the direct UV-B sensing UVR8 pathway. Plants showed no signs of damage under either low or high fluence conditions although the increased red pigmentation made determination of visual damage difficult. This is expected as the high amounts of flavones found in these plants before UV-B irradiation would have giving them high protection against UV-B damage. Plants grown under conditions without UV-B light already had large accumulation of flavonoids in the surface layers which would have given them a greater resistance due to UV-B screening when challenged at both high and low fluence treatments as compared to WT or flavone deficient mutants. The apigenin to luteolin ratio showed no significant difference in the control and high fluence treatments. However under low fluence treatment the ratio was significantly lower. This suggested that the over expression of MYB14 results in greater accumulation of luteolin flavones as compared to apigenin. This is an interesting observation as we also see that MYB14 expression is high in the 1 day samples of high and low fluence but not at 4 hour high fluence which suggests it responds not through the direct UV-B pathway but potentially through stress related activation. This is further shown in (Albert and Davies Unpublished) with increased expression under high white light and nutrient deprivation conditions. This expression under stressful conditions and the increase in luteolin flavones may indicate that MYB14 regulates ROS through the induction of ROS scavengers such as luteolin flavones and also through riccionidinA production (Albert *et al.*, 2018). An acclimatisation study of the MYB14 over-expresser showed that plants were able to grow but also in a stunted phenotype, typical of the UV-B phenotype.

*myb14* MUTANT

The *myb14* mutant showed similar response under all UV-B treatments to that of WT. No difference in the flavone response was seen to that of WT and subsequently the damage characteristics we observed were similar to WT, with little damage under low fluence and increased sensitivity and damage under high fluence conditions. This further reinforces that

MYB14 may not be directly involved in the UV-B response but rather in the stress response pathway. It would be likely that *myb14* plants may be damaged greater than WT plants in high fluence UV-B as *myb14* plants may be lacking in stress response products such as riccionidinA, yet we see only a slight enhanced damage likely due to the flavone response still able to defend plants before the need for the stress response pathway activation. Analysis of the *myb14* mutant and over-expressor show that this MYB may not be regulated directly through UV-B but may be important for the production of the red pigment riccionidinA and in the generic stress response. This is further shown by its analysis in high light and nutrient stress situations which induce riccionidinA but not flavone (Albert and Davies Unpublished).

RUP1

*rup1* mutation resulted in high levels of flavones in the thallus tissue. This is possibly due to the enhanced activation of the UVR8 pathway as RUP1 is unable to recycle UVR8 to the inactive form and thus over stimulation occurs through the UVR8 pathway. This is illustrated by the enhanced levels of total flavones in all treatments. The ratio of apigenin to luteolin was significantly higher in all treatments compared to WT. As *rup1* activates the UVR8 pathway which leads to activation of the flavonoid pathway it may be that activation of the flavonoid pathway through UVR8 alone results in high production of apigenin. This further supports our analysis of the high and low response in WT *M. polymorpha* whereby the response to high fluence, which may not be fully dependent on the UVR8 pathway, results in higher amounts of luteolin flavones while low fluence UVR8 activation results in higher amounts of apigenin. Apigenin may be preferentially produced through the UVR8 pathway and other regulators that may be activated in response to stress such as MYB14 may increase production or regulate the conversion to luteolin. When whole plants were analysed for the effects of UV-B irradiance no damage characteristics were seen at high or low fluence which suggests that the increase in flavone response in *rup1* mutants results in increased defence against UV-B. In particular high fluence plants showed no difference to that of the control treatment indicating very strong defence. However acclimation treatment with prolonged low UV-B resulted in very severe stunting. This stunting is also seen in constitutively active UVR8 Arabidopsis plants under UV-B conditions (Heijde *et al.*, 2013).

This stunting is likely due to the miss-regulation of flavonoid compounds during early growth. However *rup1* mutants demonstrate the importance of the UVR8 pathway and its associated production of flavones in the protection of *M. polymorpha* under UV-B conditions. *rup1* plants were also seen to have elevated levels of red pigment in the thallus, which is suggested to be riccionidinA. This increased production could be due to the up-regulation of the flavonoid pathway via UVR8 resulting in riccionidinA production. Its increased accumulation in *M. polymorpha* under UV-B may be the result of a general increase in flux through the flavonoid pathway rather than a direct result of UV-B induction. This is further shown by the high fluence treatment which induces higher accumulation of riccionidinA in *rup1* mutants and also in the 35s:MYB14 over expression line, which strongly indicates that riccionidinA functions as a stress related compound.

Overall we are able to show that elevated levels of flavones are able to protect *M. polymorpha* against damaging UV-B and that the loss of these flavones results in hypersensitivity to UV-B. In particular loss of flavones through the *chi* mutant results in stunted plants and a hypersensitive response upon UV-B irradiation. Alternatively constitutive activation of the flavone pathway through *rup1* mutation results in increased flavone compound accumulation and resistance to UV-B damage. These findings support our hypothesis that flavones are an important compound in the defence of *M. polymorpha* to UV-B irradiation.

ROS accumulation

Flavones are known to function in the scavenging of ROS within plants so the effect of reduced or enhanced flavone production on ROS accumulation was analysed through high fluence treatment of plants with modified flavone profiles (Fig. 6.11). It was seen that the loss of flavones resulted in high accumulation of ROS within the thallus tissue particularly within the chloroplasts. Conversely the enhancement of flavones levels in plants resulted in the loss of ROS accumulation in the chloroplasts. This difference strongly indicates that flavones provide a role in regulating the ROS homeostatis in plants. The mode of action may be hard to determine however, as both UV-B screening and ROS scavenging could be used to reduce ROS formation. *chi* plants had reduced flavones in the epidermal layers which would give very little UV-B screening. As such, tissues are vulnerable to UV-B, and ROS

accumulation may largely be due to this loss of screening. Once ROS are produced the lack of flavones in the cells may mean they are unable to scavenge them and subsequently we observe the high accumulations of ROS. In comparison *rup1* mutants have high levels of flavones in epidermal layers, indicating a large UV-B screening function. The lack of ROS in these plants may be attributed both to the enhanced UV-B screening reducing incident UV-B from entering lower tissue and also the ROS scavenging ability of flavones within the cell preventing ROS accumulation. It must be noted that *chi* plants exhibit enhanced damage in response to challenge by UV-B and are severely stunted in acclimation tests, which may be caused by the miss-regulation of ROS which damages cellular processes (Jordan, 2017). In comparison, *rup1* plants show no signs of damage upon UV-B challenge but are also severely stunted in acclimation tests. However, this stunting may not be attributed to the production of ROS, and plants are possibly stunted due to over production of flavones in early development, although this would take further research to confirm. The contribution to protection of the epidermal layer versus the mesophyll cells with respect to UV-B protectant has been previously measured in *Secale cereale* (Burchard *et al.*, 2000). It was found that while the epidermal layers were enhanced in flavonoids in an age and irradiation dependent manner the level of mesophyll flavonoids remained at a steady state. This would indicate that UV-B screening is preferential in response to UV-B in this plant for protection against UV-B. In comparison, we also see high accumulation of flavonoids in response to UV-B in the epidermal layers of *M. polymorpha,* which suggests similar protective function. While mutant analysis has shown the importance of flavones in protection against UV-B the importance of epidermal or mesophyll localisation is not definitively analysed in this study. Epidermal removal or mutation of flavonoid transporters would be an interesting method in order to analyse the protective contribution based on localisation. We also propose that luteolin-based flavones are responsible for a larger ROS scavenging activity while apigenin is involved in UV-B screening. If the flavone profile could be modified to result in production of apigenin or luteolin alone this may provide a better understanding for the importance of each, whether that be UV-B screening or ROS scavenging.

# Chapter 7: nCounter analysis of M. polymorpha genes under low fluence UV-B conditions

## 7.1: Introduction

A range of gene candidates involved in the response of *M. polymorpha* to UV-B were found using RNA-sequencing including those from the UVR8, flavonoid biosynthesis and general stress response pathways. Mutational analysis of key genes involved in the UV-B response such as *hy5*, *rup1* and over-expression of UVR8 resulted in modification of the flavonoid profile and acclimation response. Each of these mutations may affect flavonoid production in different ways and regulation through different partners so in order to further understand how *M. polymorpha* responds to UV-B, nCounter analysis on a range of genes of interest was conducted. Time points were at 12 hours and 36 hours, which corresponded to the end of day one and day two UV-B exposure. The UV-B level used for these treatments was that of the low fluence treatment.

## 7.2 Results

### 7.2.1 Analysis of UVR8 pathway expression under UV-B conditions

Candidate genes for the direct UVR8 pathway had previously been found and so the analysis of key regulator genes was used for nCounter of each *M. polymorpha* line. Significant differences are indicated as statistically different from WT response. No significant difference in UVR8 expression was found between WT, *hy5* and *rup1* mutants under any time point, although, the UVR8 over-expression line had high levels of UVR8 transcript (Fig. 7.1).

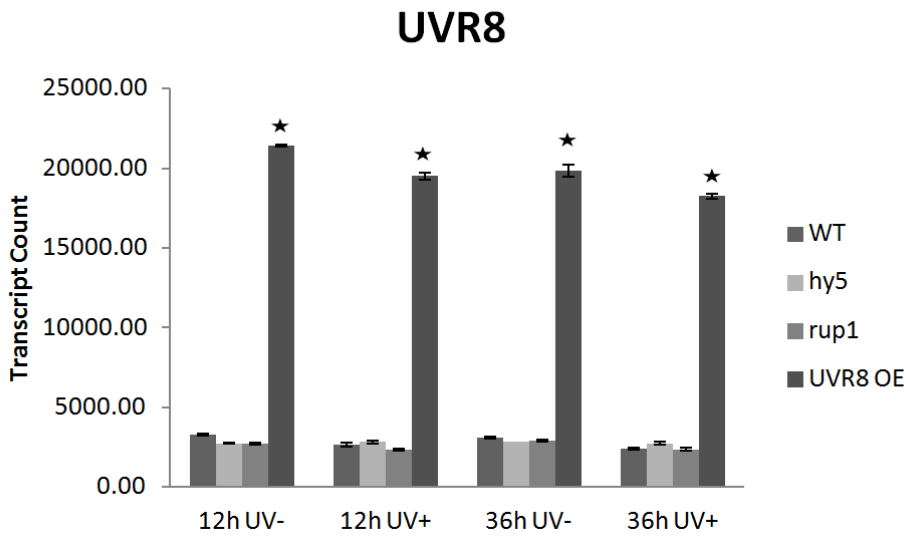



Figure 7.1: UVR8 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). UVR8 gene model = Mapoly0023s0125.1

COP1 expression was elevated in response to UV-B in the WT plants at both 12 and 36 hours of UV-B treatment as compared to the WT UV-B lacking control. The *hy5* mutant did not shown any significant difference from WT except at 12 hours under UV-B lacking conditions, when transcript levels were decreased. The response to UV-B in the *hy5* mutant followed the trend of WT with an increase in response to UV-B at both 12 and 36 hours. The *rup1* mutant had significantly higher COP1 transcript abundance at 12 and 36 hours under both UV-B and UV-B lacking conditions. Similarly UVR8 OE plants had significantly higher transcript counts at all time points (Fig. 7.2). While the amount of transcript may have showed significant differences the fold change of UV-B treated plants as compared to UV-B lacking controls showed a similar response in all lines tested of around a 2 fold increase (Fig. 7.2).

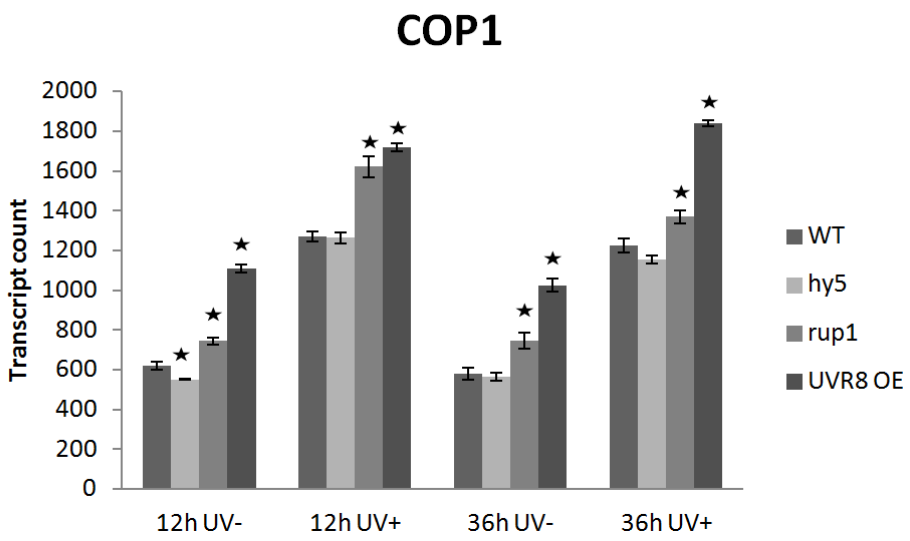



Figure 7.2: COP1 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). COP1 gene model = Mapoly0143s0030.1

RUP1 expression increased in response to UV-B irradiation at both 12 and 36 hours as compared to the UV-B lacking control (Fig.7.3). *hy5* mutants had significantly lower RUP1 transcript abundance at 12 hours under UV-B treatment and both UV-B and UV-B lacking conditions at 36 hours as compared to WT. *rup1* mutants showed no difference in response from WT at 12 hours. At 36 hours *rup1* mutants showed significantly lower transcript counts. The UVR8 OE had significantly higher RUP1 transcript abundance at all time points as compared to WT. RUP1 expression in UVR8 OE plants also increased in response to UV-B (Fig. 7.3).

SPA1 expression increased in response to UV-B at both 12 and 36 hours (Fig. 7.4). SPA1 expression showed no significant change in the plant mutant and over-expressing lines under any time point as compared to WT (Fig. 7.4).

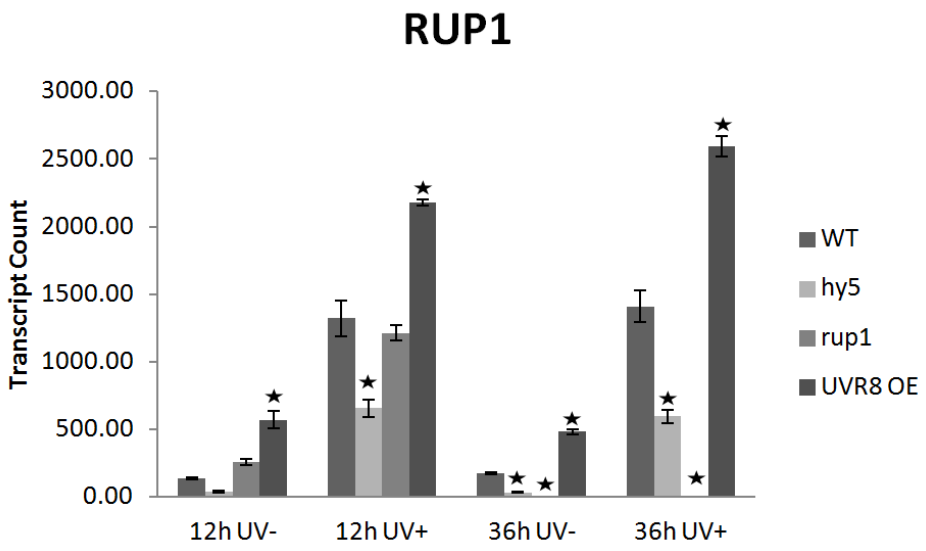



Figure 7.3: RUP1 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT (P< 0.05). Error bars = standard error (n=3). RUP1 gene model = Mapoly0094s0072.1

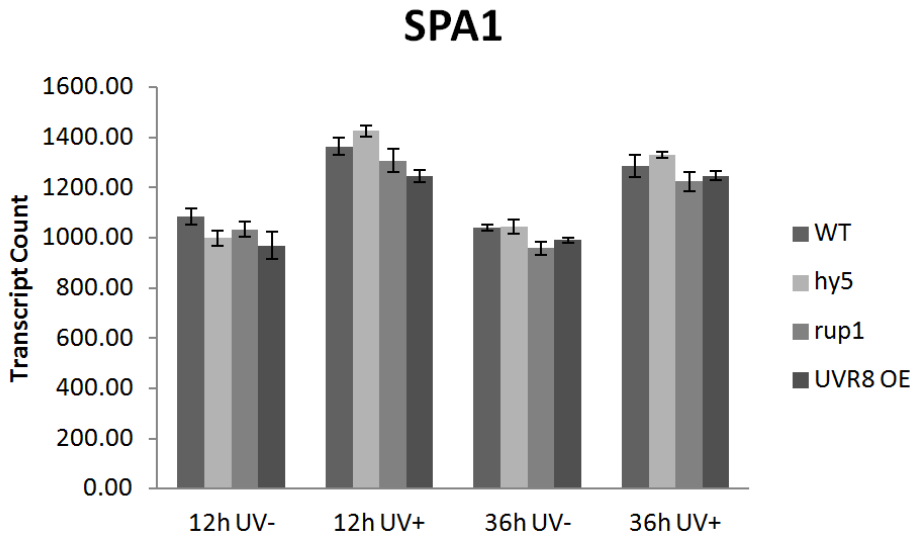



Figure 7.4: SPA1 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT (P< 0.05). Error bars = standard error (n=3). SPA1 gene model = Mapoly0100s0059.1

HY5 transcript abundance increased in response to UV-B at both 12 and 36 hours in WT (Fig. 7.5). HY5 transcript levels in *hy5* mutants were significantly lower than WT but also showed slight increase from very low levels in response to UV-B. In *rup1* mutants HY5 transcript was significantly higher in 12 hour samples under both UV-B and UV-B lacking treatment. HY5 transcripts were lower in *rup1* plants at 36 hours with UV-B, but showed no difference at 36 hours in UV-B lacking conditions. UVR8 OE plants showed significantly higher transcript at all time points as compared to WT (Fig. 7.5).

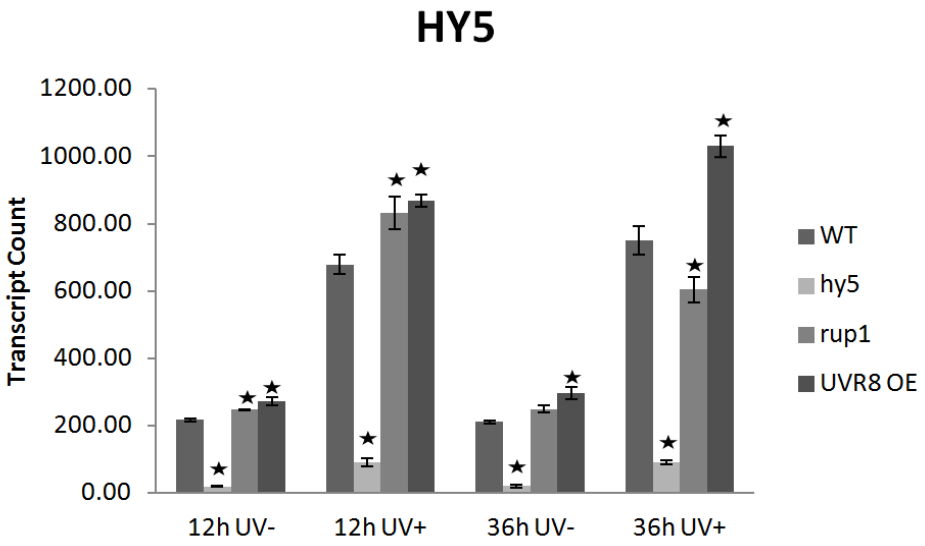



Figure 7.5: HY5 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT (P< 0.05). Error bars = standard error (n=3). HY5 gene model = Mapoly0001s0021.1

SUB1 transcript counts increased in WT in response to UV-B at both 12 and 36 hours (Fig. 7.6). *hy5* mutants plants showed elevated levels of SUB1 transcript at 12 hours with and without UV-B and also at 36 hours without UV-B. At 36 hours with additional UV-B no change was seen between WT and *hy5* mutants in SUB1 transcript level. r*up1* mutants showed no difference to WT under UV-B lacking conditions at both 12 and 36 hours. However, transcript levels for SUB1 were significantly lower in *rup1* mutants at 12 and 36 hours with addition UV-B. UVR8 OE plants showed significantly lower levels of SUB1 transcript at all time points and UV-B conditions (Fig. 7.6).

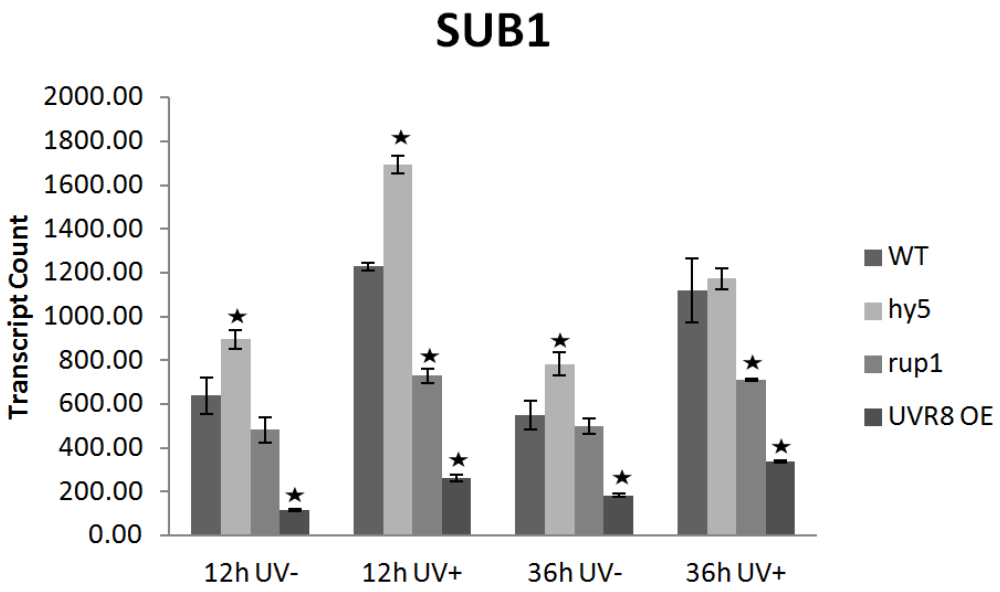



Figure 7.6: SUB1 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT (P< 0.05). Error bars = standard error (n=3). SUB1 gene model = Mapoly0016s0050.1

### 7.2.2 Analysis of putative flavonoid transcription factors

Putative transcription factors that had previously been seen to be differentially expressed in response to UV-B were analysed by nCounter to determine how the expression may differ in *hy5*, *rup1*, and UVR8 OE lines.

MYB14 expression increased under UV-B conditions at both 12 and 36 hours in WT (Fig. 7.7). MYB14 expression in *hy5* mutants was significantly higher at 12 hours with UV-B and 36 hours lacking UV-B as compared to WT. No significant difference was seen at 12 hours without UV-B and 36 hours with UV-B as compared to WT. *rup1* mutants had no significant difference in transcript level at 12 and 36 hours under UV-B lacking conditions. Transcript level was significantly reduced at 12 hours with addition of UV-B as compared to WT in *rup1* mutants. At 36 hours with addition of UV-B, while not statistically significant, we still observe a large drop in transcript abundance in *rup1* mutants as compared to WT. UVR8 OE plants show significantly lower levels of MYB14 transcript under all time points and UV-B conditions (Fig. 7.7).

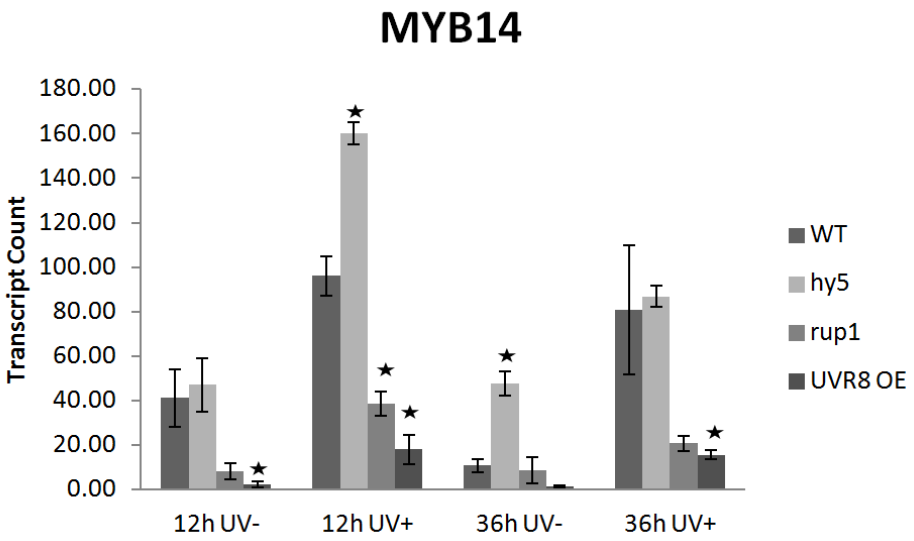



Figure 7.7: MYB14 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). MYB14 gene model = Mapoly0073s0038.1

MYB2 transcript levels were unchanged in response to UV-B in WT and the *hy5* mutant at 12 and 36 hours (Fig. 7.8). *rup1* mutants had no significant difference in MYB2 transcript level at 12 hours without UV-B but had lower transcript abundance at all other time points with and without UV-B. UVR8 OE plants showed MYB2 transcript levels that were significantly lower at all time points and UV-B conditions (Fig. 7.8).

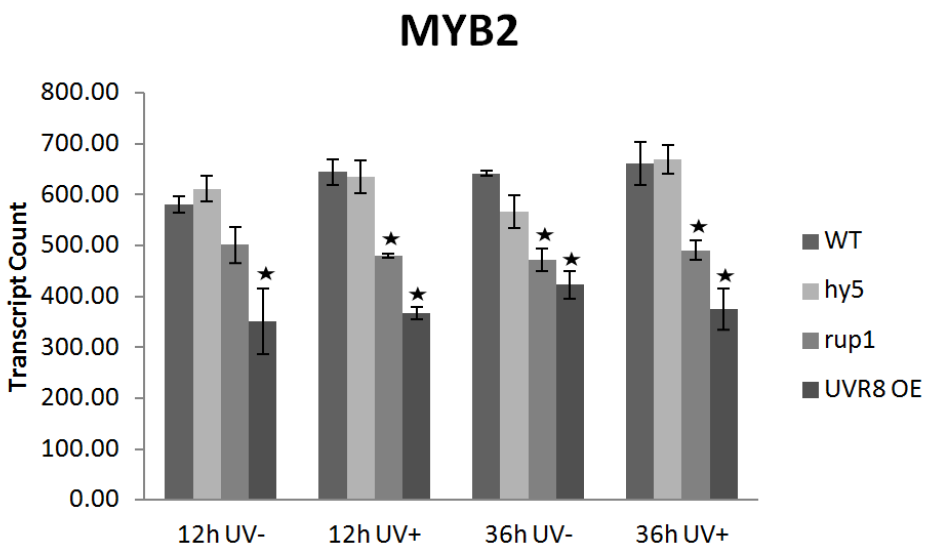



Figure 7.8: MYB2 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). MYB2 gene model = Mapoly0006s0226.1

An unknown gene labelled UNK2 was also analysed for its expression. It had previously been observed to be highly up-regulated in high fluence treatment conditions. UNK2 expression was slightly elevated in response to UV-B at 12 and 36 hours in WT plants. *hy5* mutant plants had significantly higher transcript abundance at all time points with and without UV-B as compared to WT. *rup1* mutant plants had no significant difference in UNK2 transcript level at 12 and 36 hours under both UV-B and UV-B lacking conditions as compared to WT. UVR8 OE plants had significantly lower levels of UNK2 transcript under all time points and UV-B conditions as compared to WT (Fig. 7.9).

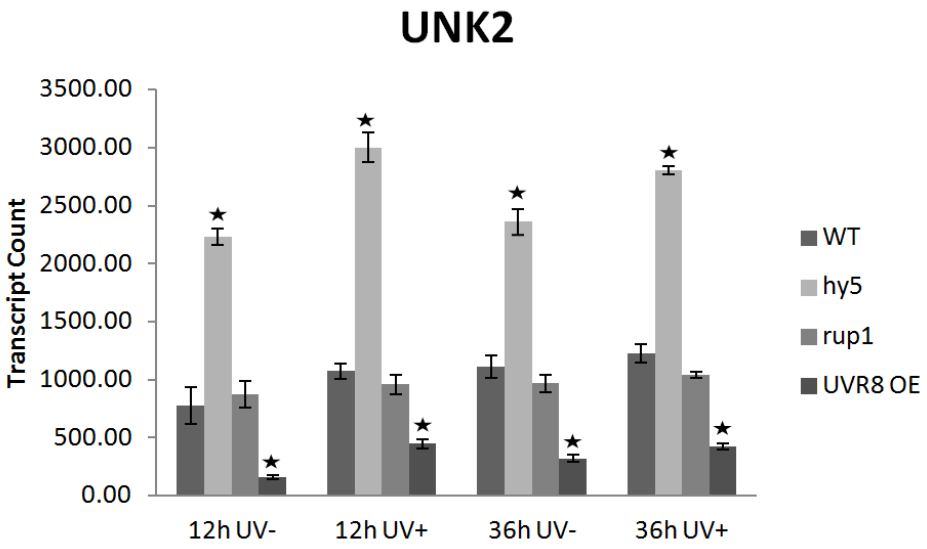



Figure 7.9: UNK2 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). UNK2 gene model = Mapoly0039s0049.1

Previously identified as a WRKY interacting transcription factor a putative VQ motif gene was also analysed due to previously identified increased expression under UV-B conditions. Transcript levels increased under low fluence treatment at both 12 and 36 hours in response to UV-B (Fig. 7.10). No significant difference was observed in *hy5* mutants under any time point or UV-B condition as compared to WT. *rup1* mutants had significantly lower transcript level at 12 and 36 hours with and without UV-B as compared to WT. UVR8 OE plants had significantly lower transcript levels at 12 hours with and without UV-B and 36 hours with UV-B as compared to WT (Fig. 7.10).

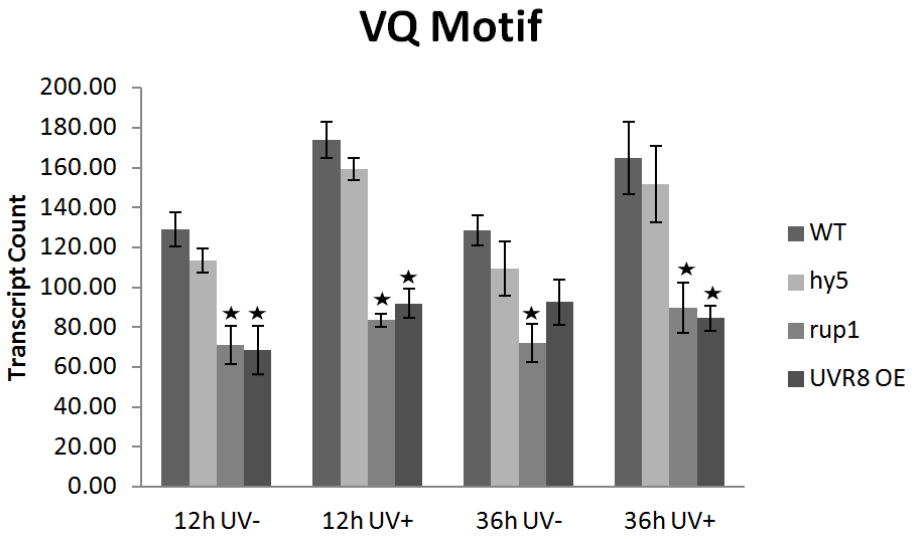



Figure 7.10: VQ motif nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). VQ motif gene model = Mapoly0044s0085.1

GLABRA 3 expression was previously seen to be elevated at 1 day in response to UV-B at both high and low UV-B fluence. Measurement of GL3 expression at 12 and 36 hours showed minor increases in response to UV-B low fluence treatment (Fig. 7.11). *hy5* mutants showed no significant difference in GL3 transcript at 12 hours with and without UV-B as compared to WT. At 36 hours *hy5* mutants showed significantly lower levels of GL3 transcript under UV-B lacking conditions but no difference under UV-B conditions as compared to WT. *rup1* mutants and UVR8 OE plants had significantly lower levels of GL3 transcript at all time points and UV-B conditions (Fig. 7.11).

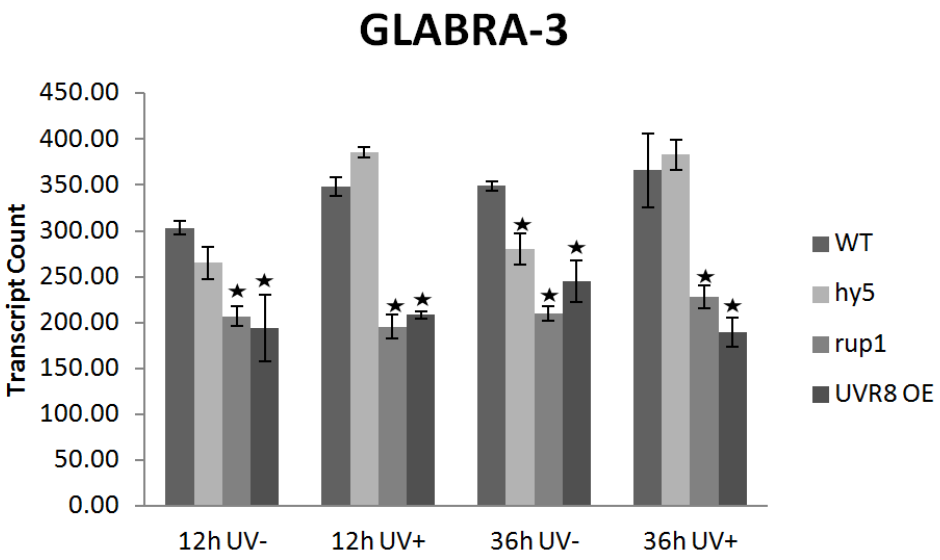



Figure 7.11: GLABRA-3 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT (P< 0.05). Error bars = standard error (n=3). GLABRA-3 gene model = Mapoly0028s0058.1

GLABRA-3-like expression was seen to increase in response to UV-B in WT plants at both 12 and 36 hours (Fig. 7.12). No significant differences were observed as compared to WT except for that of the *rup1* mutants at 36 hours of UV-B lacking conditions where a significant, but small, increase in GL3-like transcript was observed (Fig. 7.12).

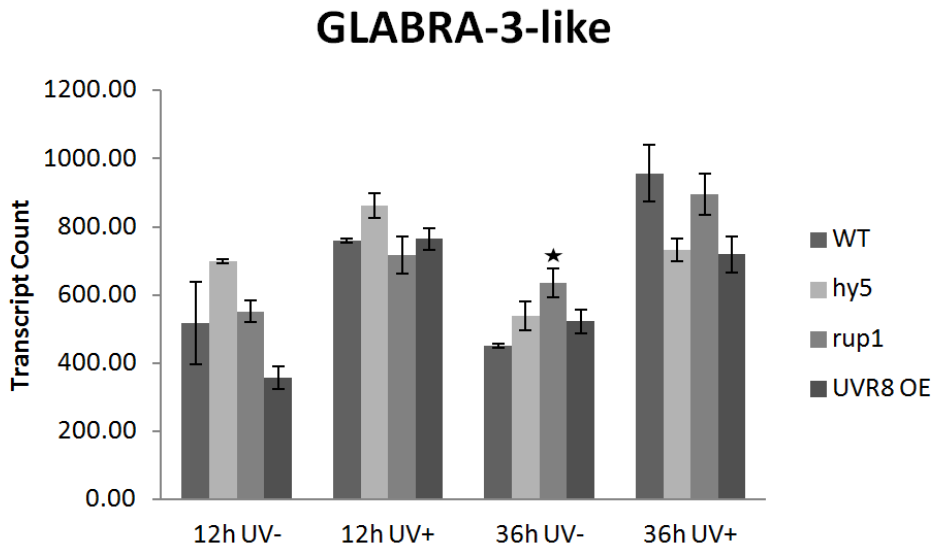



Figure 7.12: GL3-like nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT (P< 0.05). Error bars = standard error (n=3). GL3-like gene model = Mapoly0028s0060.1

### 7.2.3 Analysis of flavonoid biosynthetic genes

The flavonoid biosynthetic genes were shown to be up-regulated in response to UV-B in RNA-seq data under high and low fluence. nCounter analysis was used on flavonoid regulator mutant and over-expression lines to determine the effect on the flavonoid biosynthetic genes. PAL3 transcripts were elevated in UV-B conditions at 12 and 36 hours for WT plants. *hy5* mutants had elevated PAL3 transcript at all time points and UV-B conditions as compared to WT. *rup1* mutants had lower levels of PAL3 transcript but only at 12 hours under UV-B was this difference statistically significant as compared to WT. UVR8 OE plants had significantly lower PAL3 transcript at all time points and UV-B conditions (Fig. 7.13).

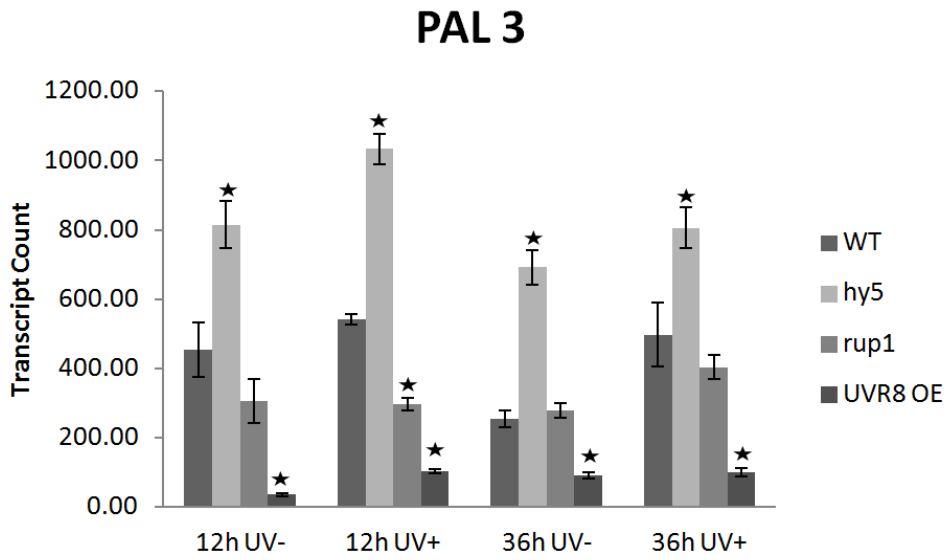



Figure 7.13: PAL3 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). PAL3 gene model = Mapoly0009s0173.1

PAL4 transcripts increased under UV-B conditions at both 12 and 36 hours in WT plants (Fig. 7.14). *hy5* mutants had significantly lower PAL4 transcript at 12 hours with and without UV-B and 36 hour with UV-B. No significant difference in PAL4 transcript at 36 hours without UV-B was observed in *hy5* mutants. *rup1* mutants had significantly higher PAL4 transcript at 12 hours with and without UV-B. At 36 hours PAL4 transcript was significantly lower without UV-B but not significantly different with addition of UV-B as compared to WT. UVR8 OE

plants had elevated PAL4 transcript under 12 and 36 hours with addition of UV-B as compared to WT. At 12 hours UVR8 OE plants had significantly lower levels under UV-B lacking conditions and no significant change at 36 hours when lacking UV-B (Fig. 7.14).

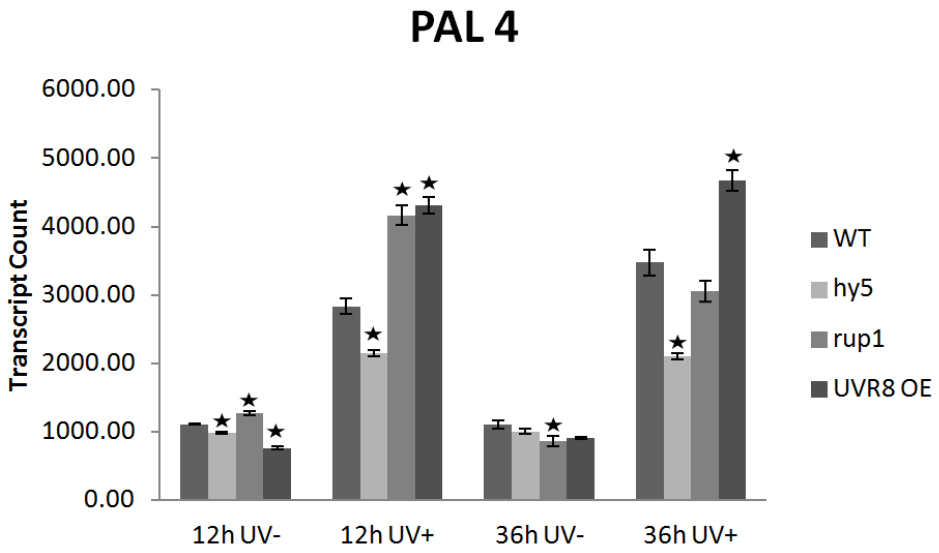



Figure 7.14: PAL4 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). PAL4 gene model = Mapoly0014s0211.1

CHS candidates were also tested to determine the effect on transcript counts in mutant and over-expression lines. The gene CHS 21-159 showed high up-regulation in response to UV-B at both 12 and 36 hours in WT (Fig. 7.16). *hy5* mutants had significantly lower CHS 21-159 transcript levels at 12 and 36 hours under both UV-B and UV-B lacking conditions as compared to WT. *rup1* mutants had elevated CHS 21-159 transcript at 12 hours with and without UV-B as compared to WT. CHS 21-159 transcript in *rup1* mutants was significantly higher at 36 hours without UV-B but showed no difference to WT with addition of UV-B at 36 hours. UVR8 OE plants had significantly higher CHS 21-159 transcript in response with and without UV-B at all time points as compared to WT (Fig. 7.16).

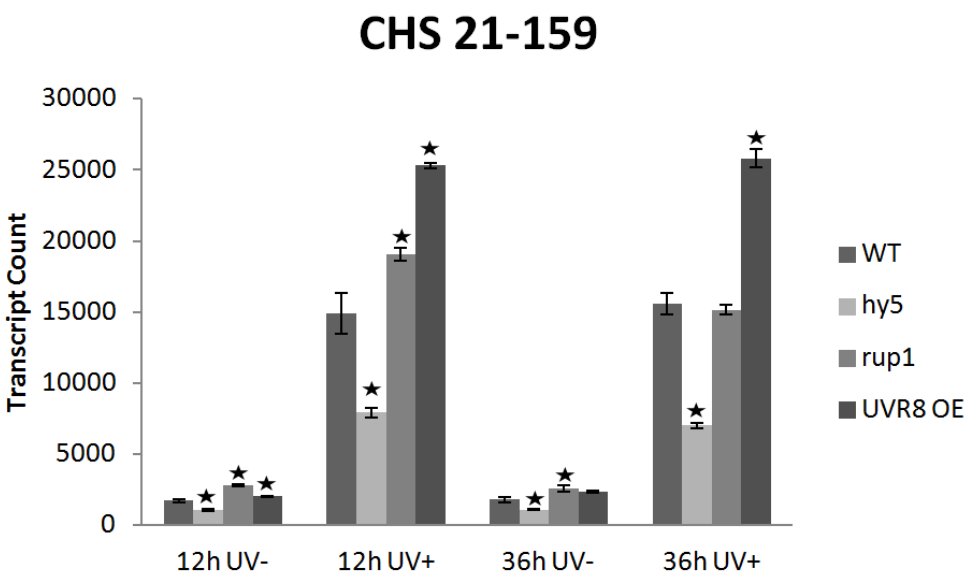



Figure 7.16: CHS 21-159 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). CHS 21-159 gene model = Mapoly0021s0159.1

A second CHS candidate was analysed and WT plants showed a decrease in transcript at 12 hours in response to UV-B and an increase in transcript at 36 hours with low fluence UV-B (Fig. 7.15). *hy5* mutants showed no significant change in CHS 70-36 gene transcript at any time point except for 12 hours with the addition of UV-B where and increased level of transcript was observed. *rup1* mutants and UVR8 OE plants had significantly lower CHS 70-36 transcript at each time point except for at 36 hours without UV-B where a decrease was still observed but not statistically significant (Fig. 7.15).

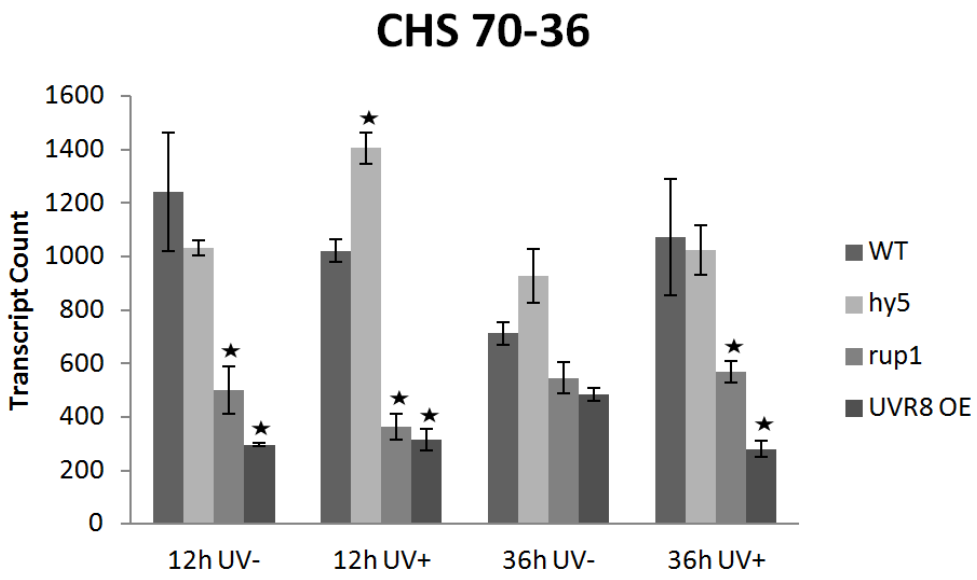



Figure 7.15: CHS 70-36 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT (P< 0.05). Error bars = standard error (n=3). CHS 70-36 gene model = Mapoly0070s0036.1

CHI transcripts increased under UV-B conditions at both 12 and 36 hours in WT plants (Fig. 7.17). *hy5* mutants showed no significant difference in response in CHI transcript from that of WT. *rup1* mutant plants had significantly higher CHI transcripts at 12 hours with and without UV-B as compared to wild type. At 36 hours without UV-B no significant difference in CHI transcript between WT and *rup1* mutants was observed. At 36 hours under UV-B conditions however, *rup1* mutants showed significantly higher levels of CHI transcripts as compared to WT. UVR8 OE plants showed no significant changes in CHI transcript amounts as compared to WT in UV-B lacking conditions. Under UV-B UVR8 OE plants at 12 and 36 hours had significantly higher levels of CHI transcripts as compared to WT (Fig. 7.17).

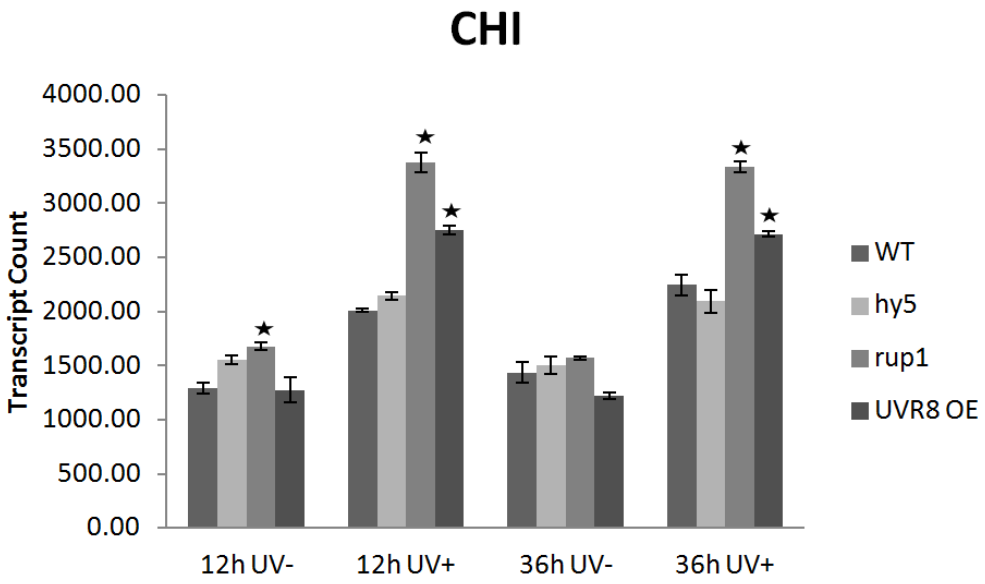



Figure 7.17: CHI nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT (P< 0.05). Error bars = standard error (n=3). CHI gene model = Mapoly0167s0012.1

CHI-L transcript levels increase with UV-B treatment at both 12 and 36 hours (Fig. 7.18). *hy5* mutants had significantly lower levels of CHI-L transcript at 12 hours with and without UV-B. However, at 36 hours without UV-B no significant difference was observed. At 36 hour with UV-B treatment *hy5* mutants had significantly lower CHI-L transcript as compared to WT. UVR8 OE plants had significantly higher CHI-L transcripts under UV-B conditions at 12 and 36 hours as compared to WT (Fig.7.18).

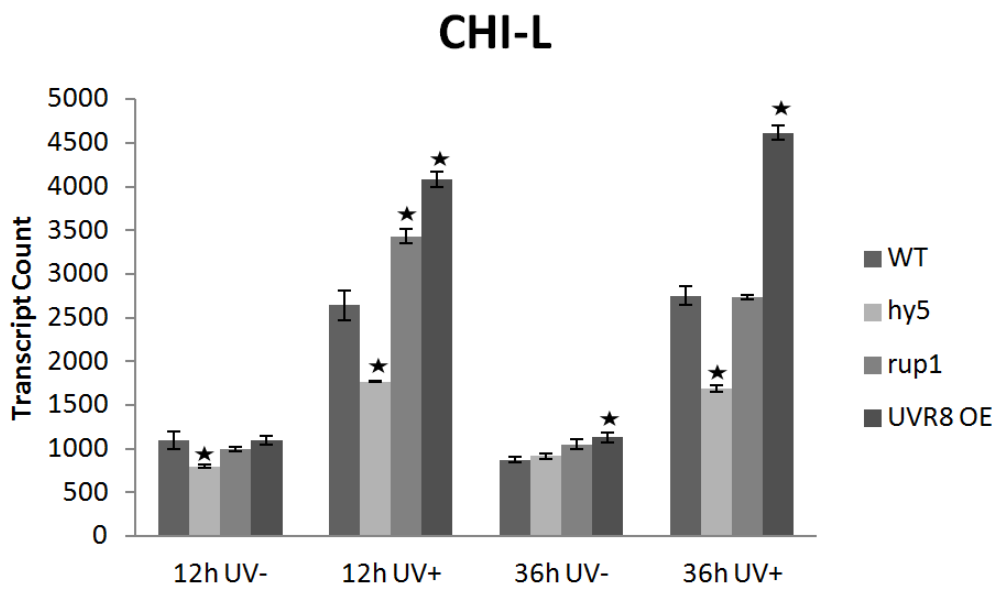



Figure 7.18: CHI-L nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). CHI-L gene model = Mapoly0175s0004.1

A dioxygenase gene (DOX) that matched closely to FNS/FNR was also studied for its regulation by UV-B. The candidate DOX 28 showed increases in transcript level under UV-B conditions at both 12 and 36 hours in WT plants (Fig. 7.19). *hy5* mutants showed significantly lower transcript levels of DOX 28 at 12 and 36 hours under UV-B conditions while no significant difference was seen under UV-B lacking conditions as compared to WT. *rup1* mutants had significantly higher DOX 28 transcript at 12 hours with and without UV-B while at 36 hours under UV-B lacking conditions DOX 28 transcripts were higher. No significant difference in DOX 28 transcript was observed in *rup1* mutants at 36 hours with UV-B. UVR8 OE plants had significantly higher transcript levels of DOX 28 at both 12 and 36 hours with and without UV-B (Fig. 7.19).

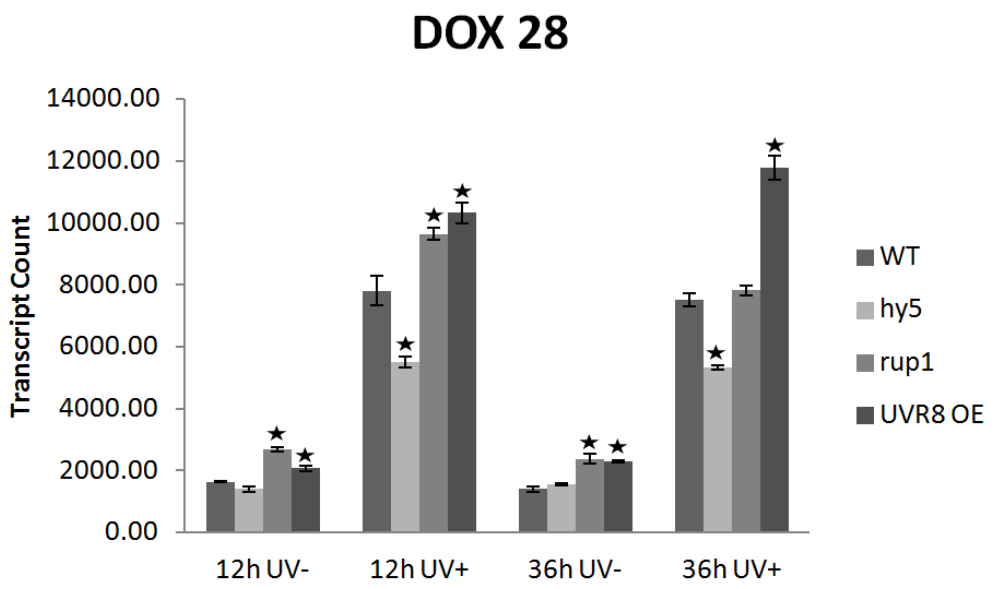



Figure 7.19: DOX 28 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). DOX 28 gene model = Mapoly0002s0224.1

A second dioxygenase gene that also matched closely to FNS/FNR was also studied for its regulation by UV-B. Transcript levels increased at 12 and 36 hours upon UV-B irradiance in WT plants (Fig. 7.20). *hy5* mutants had significantly lower levels of DOX 38 transcript at 12 hours upon UV-B treatment while no change was observed under UV-B lacking conditions at 12 hours. At 36 hours, *hy5* mutants had significantly lower levels of DOX 38 transcript with and without UV-B as compared to WT. UVR8 OE plants had significantly higher DOX 38 transcript at all time points and UV-B conditions as compared to WT (Fig. 7.20).

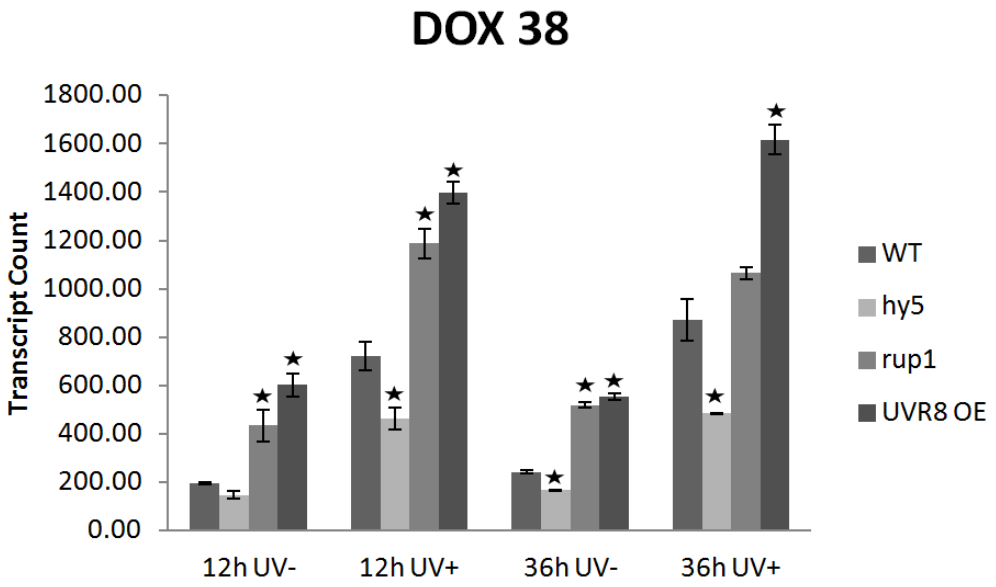



Figure 7.20: DOX 38 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). DOX 38 gene model = Mapoly0180s0025.1

### 7.2.4 Analysis of putative stress and indirect pathway genes in response to UV-B

While UV-B may directly act and induce the UVR8 pathway and flavonoid biosynthetic genes, indirect affects also occur as a stress response in *M. polymorpha*. A range of genes that may be differentially regulated in response to UV-B that act indirectly of the UVR8 pathway were analysed.

A CAB candidate CAB11-76 was analysed and showed higher transcript levels at 36 hours under UV-B conditions in WT plants (Fig. 7.21). *hy5* mutant plants had no significant changes at 12 hours but had significantly lower transcript of CAB11-76 at 36 hours with and without UV-B as compared to WT. *rup1* mutants showed no significant difference from WT under any time points or conditions. UVR8 OE plants had elevated CAB11-76 transcript at 12 hours with and without UV-B and 36 hours without UV-B as compared to WT (Fig. 7.21).

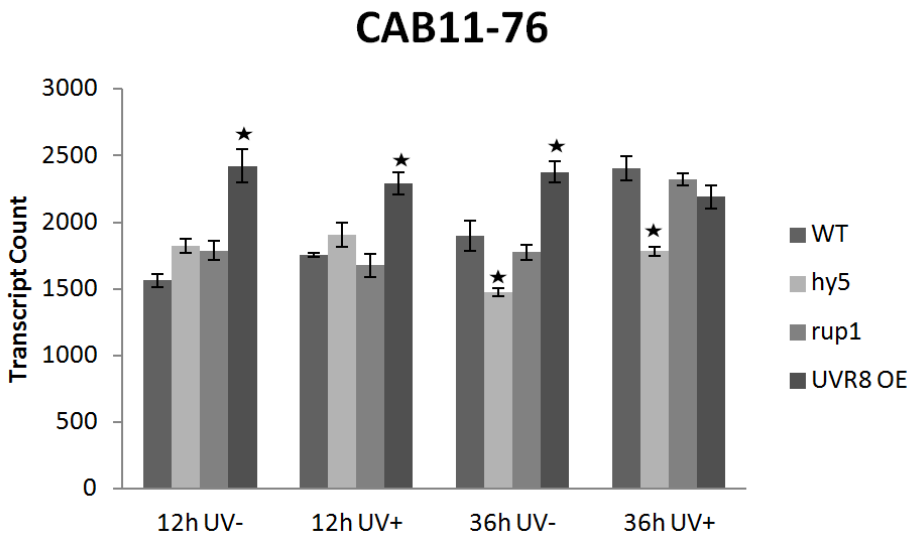



Figure 7.21: CAB11-76 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT (P< 0.05). Error bars = standard error (n=3). CAB11-76 gene model = Mapoly0011s0076.1

CAB candidate CAB6-261 was also analysed and showed higher transcript levels at 36 hours under UV-B conditions in WT plants (Fig. 7.22). *hy5* mutant plants had no significant changes at 12 hours but had significantly lower transcript of CAB11-76 at 36 hours with and without UV-B as compared to WT. *rup1* mutants showed significantly higher transcript at 12 hours without UV-B as compared to WT but no significant differences at any other time point.

UVR8 OE plants had elevated CAB11-76 transcript at 12 hours with and without UV-B and 36 hours without UV-B as compared to WT (Fig. 7.22).

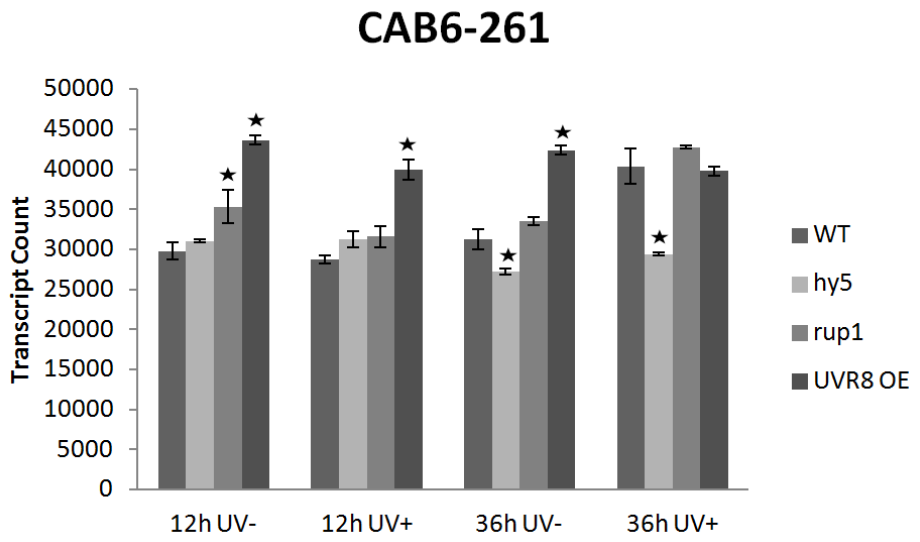



Figure 7.22: CAB6-261 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). CAB6-261 gene model = Mapoly0006s0261.1

CAB68-87 was also analysed and showed small changes in WT plants (Fig 7.23). *hy5* mutants showed significantly higher CAB68-87 transcript at 12 and 36 hours with and without UV-B. *rup1* mutants showed significantly higher transcript at 12 hours without UV-B and 36 hours with and without UV-B as compared to WT. UVR8 OE plants did not show statistically significant changes but had very little to no transcript abundance for CAB68-87 at all time points and UV-B conditions (Fig. 7.23).

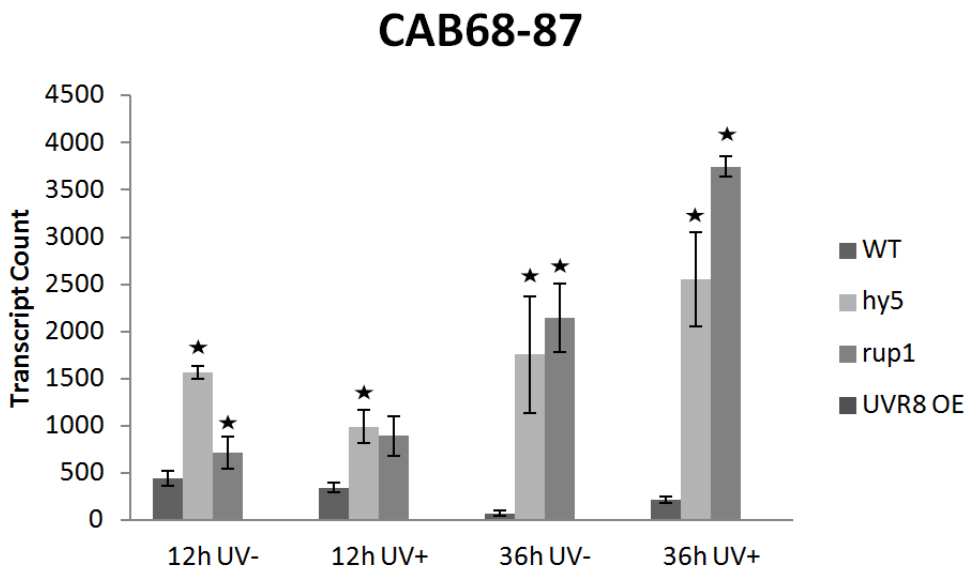



Figure 7.23: CAB68-87 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT (P< 0.05). Error bars = standard error (n=3). CAB68-87 gene model = Mapoly0068s0087.1

Gene encoding a Photosystem II protein D1 (PSII) was also analysed to investigate the stress response and showed steady state levels of transcript under low fluence conditions at 12 and 36 hours in WT (Fig. 7.24). No significant difference in PSII transcripts was observed in *hy5* mutants as compared to WT at 12 and 36 hours with and without UV-B. *rup1* mutant plants had no significant difference to WT at 12 hours without UV-B but with UV-B a significantly lower level of PSII transcript was observed. At 36 hours, with and without UV-B, *rup1* mutants had significantly lower PSII transcripts as compared to WT. UVR8 OE plants had significantly lower levels of PSII transcript over all time points and UV-B conditions (Fig. 7.24).

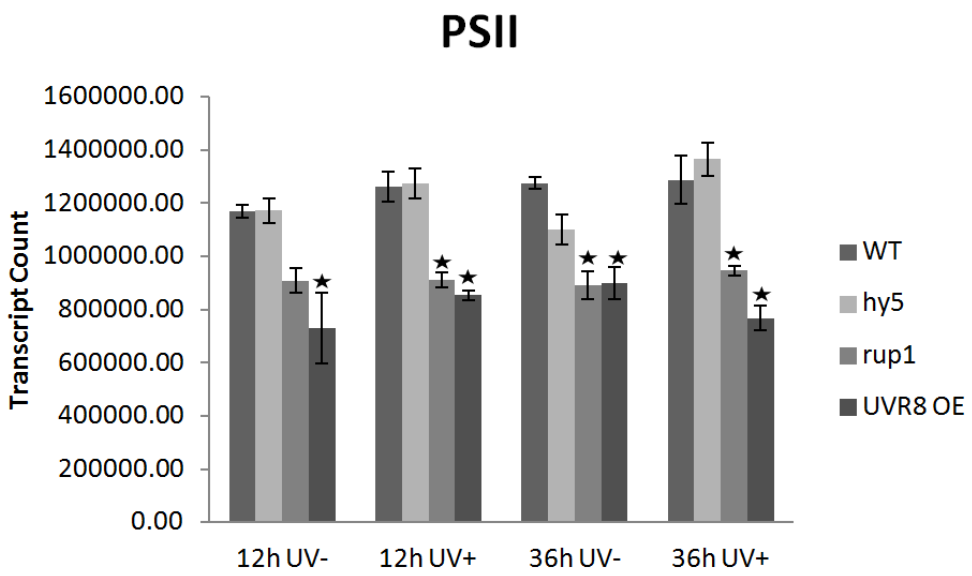



Figure 7.24: PSII nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT (P< 0.05). Error bars = standard error (n=3). PSII gene model = Mapoly0093s0016.1

ELIP genes were also analysed as they showed strong increases in transcript abundance under UV-B conditions. ELIP1 showed increases at 12 and 36 hours of low fluence UV-B (Fig. 7.25). *hy5* mutants had no significant difference in transcript levels except at 36 hours without UV-B were a higher transcript level was observed as compared to WT. *rup1* mutant plants had significantly higher transcript levels of ELIP1 at 12 hours without UV-B and 36 hours with and without UV-B as compared to WT. No significant difference was observed at 12 hours with UV-B in *rup1* or UVR8 OE plants. UVR8 OE plants had significantly higher ELIP1 transcripts at 36 hours under UV-B and UV-B lacking conditions as compared to WT (Fig. 7.25).

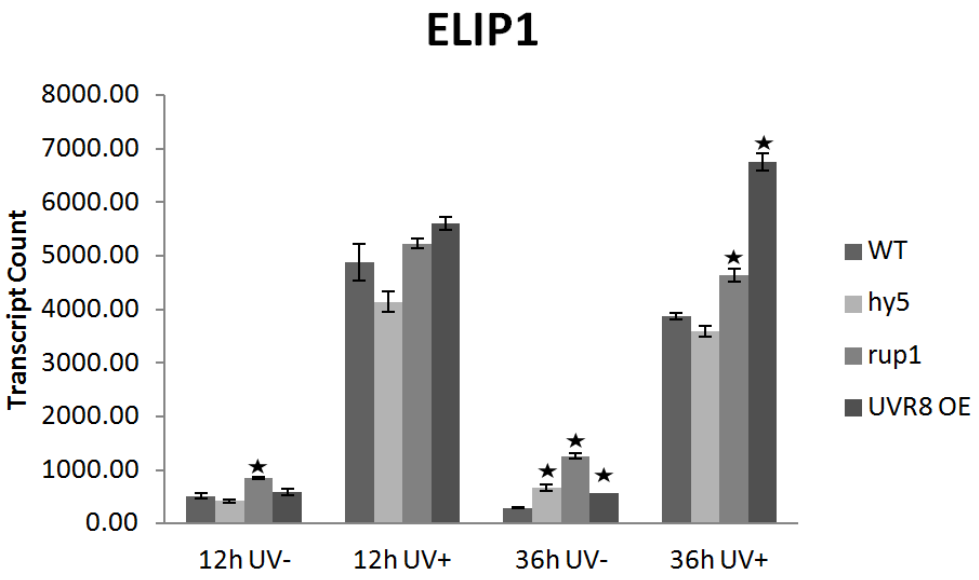



Figure 7.25: ELIP1 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT (P< 0.05). Error bars = standard error (n=3). ELIP1 gene model = Mapoly0047s0106.1

A second ELIP, ELIP2, showed moderate increases in transcription at 12 hours under UV-B but strong increases at 36 hours under UV-B conditions in WT plants (Fig. 7.26). *hy5* mutants had significantly lower ELIP2 transcript at both 12 and 36 hours with and without UV-B, as compared to WT. No other plant line had any significant difference to WT except for UVR8 OE plants which had significantly higher ELIP2 transcript at 12 hours under UV-B conditions (Fig. 7.26).

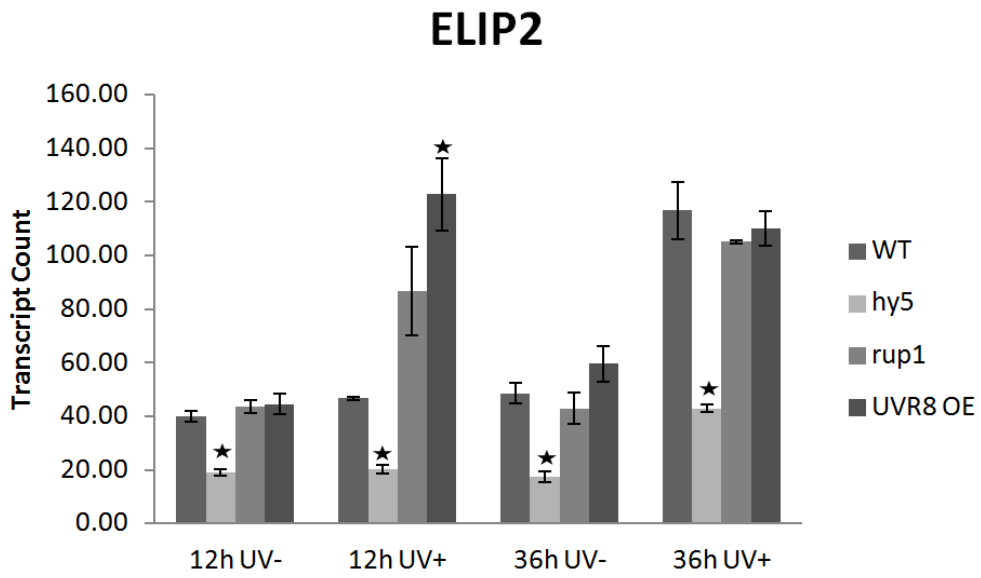



Figure 7.26: ELIP2 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). ELIP2 gene model = Mapoly0047s0108.1

Additional elements of the photosynthetic machinery may also be prone to UV-B damage and as such gene candidates for the RUBISCO small subunit were also analysed. A RUBISCOssu 114-49 was found to have higher transcript counts at 12 and 36 hours with the addition of UV-B (Fig. 7.27). *hy5* mutant plants had significantly lower transcript at 12 and 36 hours with and without UV-B. *rup1* mutants had no significant difference from WT in RUBISCOssu 114-49 transcript count. UVR8 OE plants had significantly higher levels of RUBISCOssu 114-49 transcript at 12 and 36 hours with and without UV-B (Fig. 7.27).

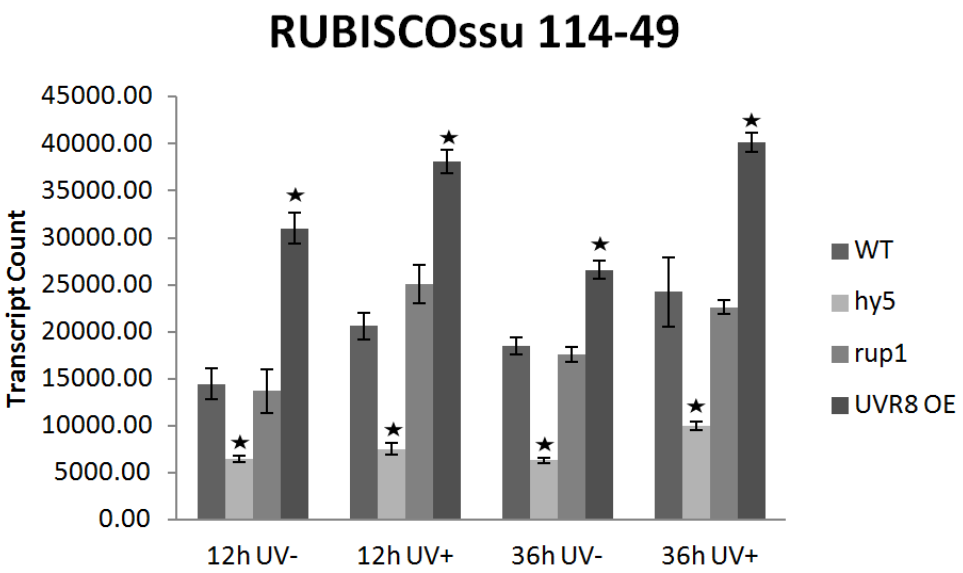



Figure 7.27: RUBISCOssu 114-49 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). RUBISCOssu 114-49 gene model = Mapoly0114s0049.1

A second RUBISCO small subunit gene, RUBISCOssu 114-53, was also analysed and showed increases in transcript abundance in response to UV-B at 12 and 36 hours in WT plants (Fig. 7.28). *hy5* plants showed no significant change in RUBISCOssu 114-53 transcript as compared to WT except at 36 hours under UV-B lacking conditions were they had a significantly lower amount. *rup1* mutant plants had no significant difference in RUBISCOssu 114-53 transcript at any time point as compared to WT. UVR8 OE plants had significantly higher RUBISCOssu 114-53 transcript counts at 12 hours with and without UV-B but no significant difference at 36 hours under either UV-B condition, as compared to WT (Fig. 7.28).

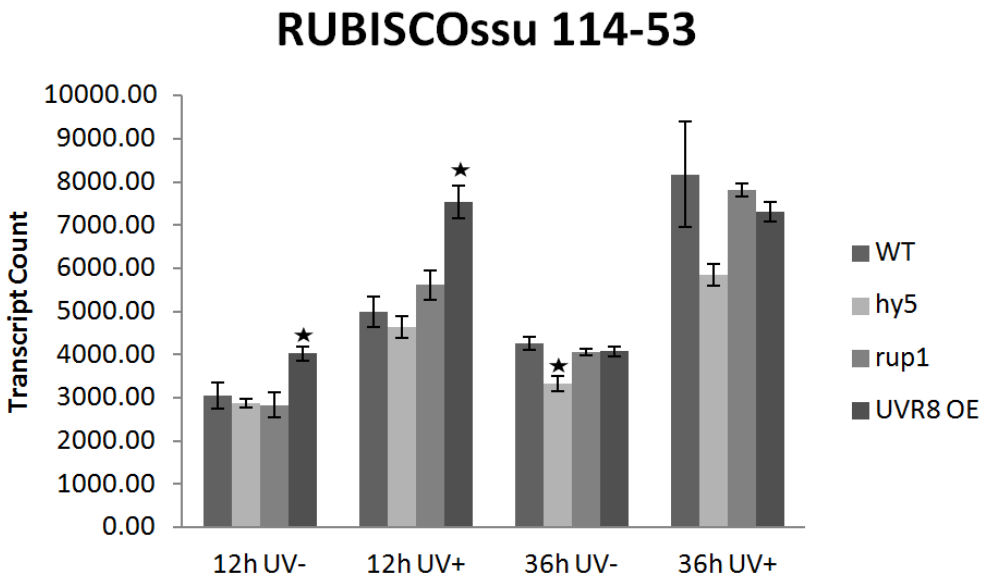



Figure 7.28: RUBISCOssu 114-53 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). RUBISCOssu 114-53 gene model = Mapoly0114s0053.1

A DNA repair candidate previously seen to be up-regulated in response to UV-B was analysed and showed increases in transcript count in response to UV-B at 12 and 36 hours in WT plants (Fig. 7.29). *hy5* mutant plants had significantly higher transcript at 12 hours with UV-B and 36 hours without UV-B. *rup1* mutant plants had significantly higher transcript at 12 hours with UV-B and 36 hours without UV-B. UVR8 OE plants had significantly higher DNA repair transcript at 12 and 36 hours with and without UV-B as compared to WT (Fig. 7.29).

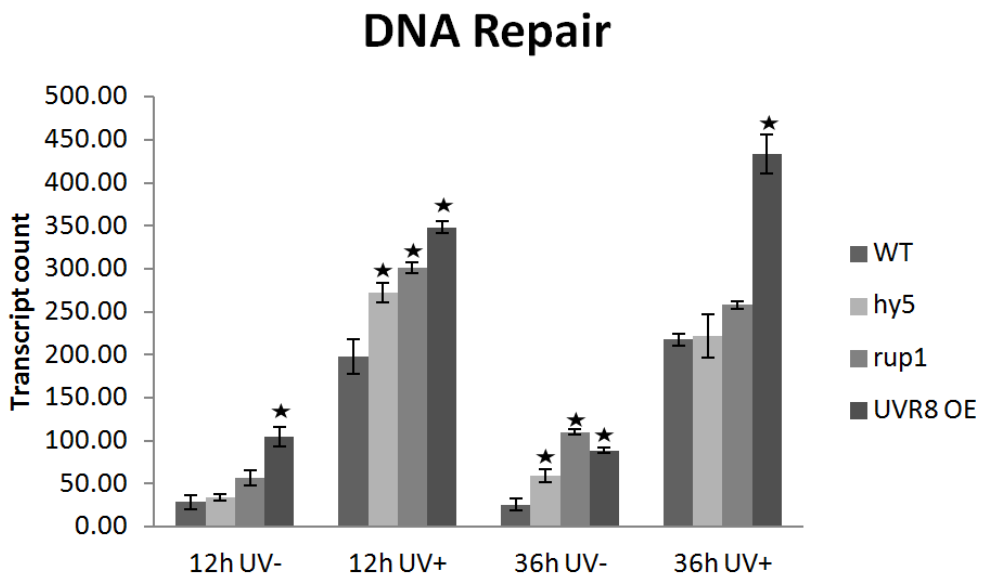



Figure 7.29: DNA repair nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). DNA Repair gene model = Mapoly0074s0054.1

DNAJ chaperone expression in WT was seen to increase at both 12 and 36 hours with addition of low fluence UV-B (Fig. 7.30). *hy5* mutant plants had no significant changes from WT transcript except for at 12 hours with the addition of UV-B where significantly higher transcripts were produced. *rup1* mutants had significantly lower transcript counts for DNAJ chaperone at 12 and 36 hours with and without UV-B. UVR8 OE plants had significantly lower transcript for DNAJ chaperone at all time points except for 36 hours without UV-B (Fig. 7.30).

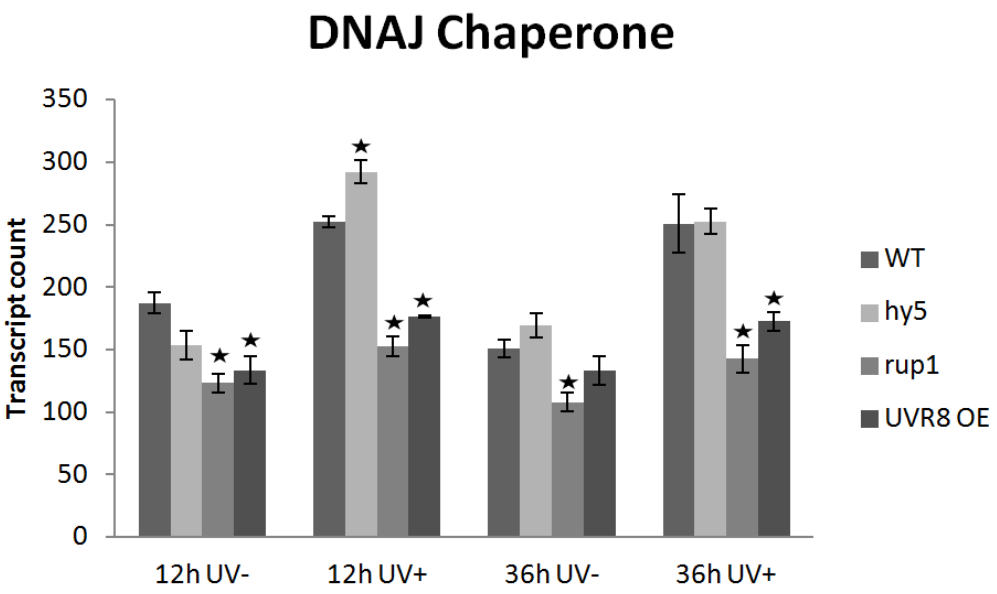



Figure 7.30: DNAJ chaperone nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). DNAJ chaperone gene model = Mapoly0088s0065.1

An ethylene response factor was also seen to be up-regulated in response to UV-B previously, and WT plants under low fluence conditions showed increases in transcript count in response to UV-B at 12 and 36 hours (Fig. 7.31). *hy5* mutants plants showed significantly higher transcript at 12 hours without UV-B, but no significant difference at 12 hours with addition of UV-B or 36 hour with and without UV-B. *rup1* mutant plants had significantly higher transcript levels at 12 hours without UV-B but significantly lower levels of ERF transcript counts at 12 hours with the addition of UV-B. At 36 hours no statistically significant difference was observed in ERF transcript count as compared to WT but with addition of UV-B *rup1* mutants had much lower ERF transcript. UVR8 OE plants had no significant changes in ERF transcript as compared to WT except at 12 hours with addition of UV-B where a significantly lower count was observed (Fig. 7.31).

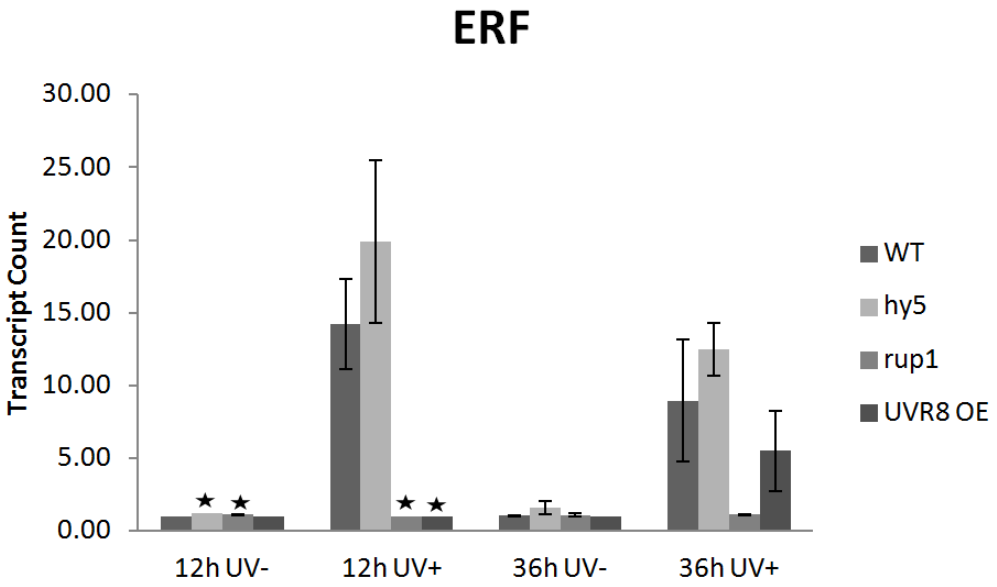



Figure 7.31: ERF nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). ERF gene model = Mapoly0166s0010.1

Heat shock protein, HSP 18, had previously been seen to be strongly up-regulated at 1 day after UV-B treatment. WT plants under low fluence showed slight increases in transcript at 12 hours with addition of UV-B and a drop in transcript at 36 hours with addition of UV-B (Fig. 7.32). *hy5* plants had significantly lower HSP 18 transcript at 12 hours with and without UV-B and 36 hours without UV-B, as compared to WT. *rup1* mutant plants had significantly lower HSP 18 transcript at 12 and 36 hours with and without UV-B. UVR8 OE plants had no significant changes in HSP 18 transcript except at 12 hours with UV-B where a lower transcript count was observed (Fig. 7.32).

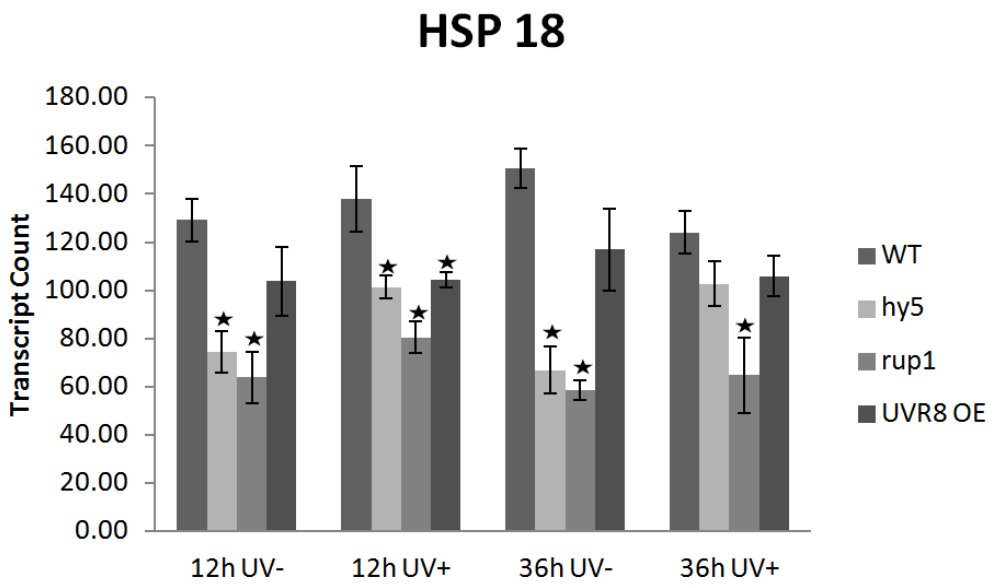



Figure 7.32: HSP 18 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). HSP 18 gene model = Mapoly0076s0006.1

A second HSP was also analysed that showed up-regulation in response to UV-B. HSP 70 showed increased transcript levels in response to low fluence UV-B at 12 and 36 hours in WT plants (Fig.7.33). No significant difference in HSP 70 transcript at 12 hours with or without UV-B was observed in *hy5* mutants. *hy5* mutants at 36 hours had significantly higher levels of HSP 70 transcript as compared to WT with and without UV-B. *rup1* mutants had no significant difference from WT plants in HSP 70 transcript count at 12 and 36 hours without addition of UV-B. At 12 and 36 hours with addition of UV-B however, *rup1* mutants had significantly lower HSP 70 transcript counts. UVR8 OE plants had significantly lower HSP 70 transcript at all time points except for 36 hours with addition of UV-B as compared to WT (Fig. 7.33).

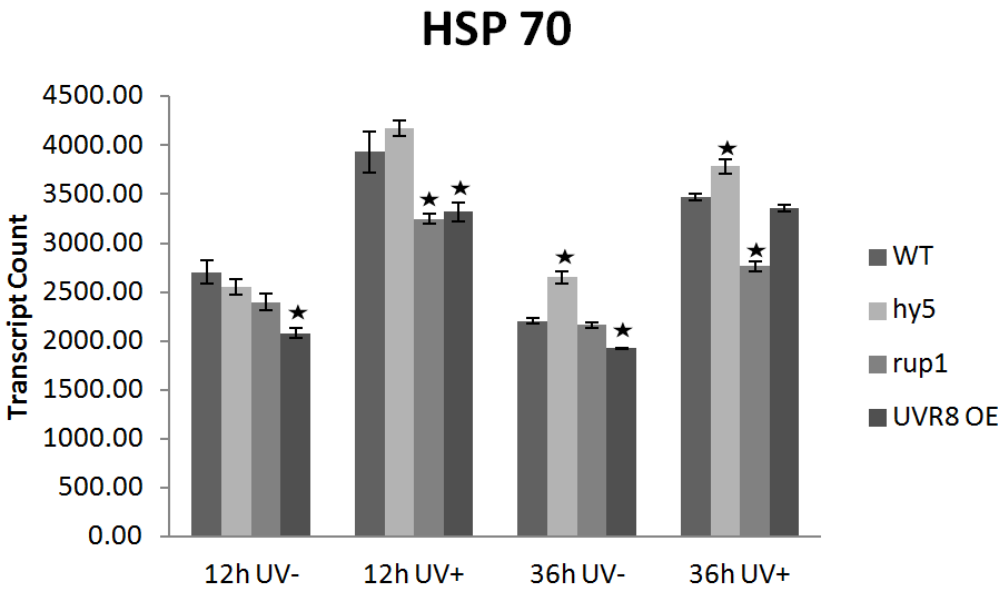



Figure 7.33: HSP 70 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). HSP 70 gene model = Mapoly0013s0060.1

Metallochaperone genes were found to be up-regulated in response to UV-B and so two gene candidates were analysed by nCounter. Metallochaperone 1 showed high up-regulation in response to UV-B low fluence at both 12 and 36 hours in WT plants (Fig. 7.34). *hy5* mutant plants had no significant change in metallochaperone 1 transcript at any time point or UV-B condition as compared to WT. *rup1* mutants had no significant change in transcript at 12 and 36 hours without UV-B but significantly lower levels of transcript at 12 and 36 hours with the addition of UV-B as compared to WT. UVR8 OE plants had significantly higher levels of metallochaperone at 12 and 36 hours without the addition of UV-B and significantly lower transcript at 12 hours with the addition of UV-B as compared to WT (Fig. 7.34).

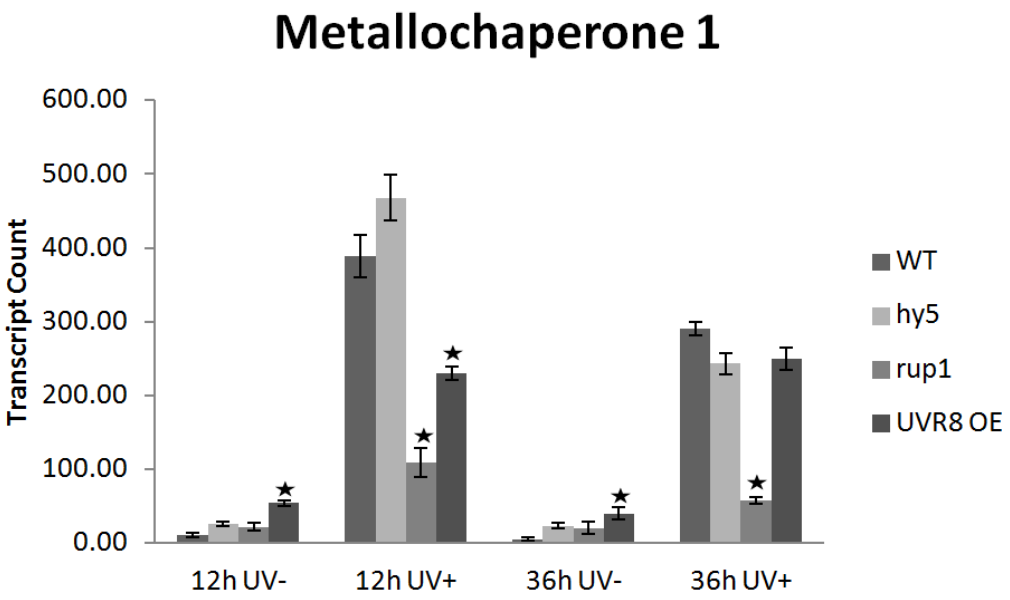



Figure 7.34: Metallochaperone 1 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). Metallochaperone 1 gene model = Mapoly0025s0089.1

A second metallochaperone showed increased transcript count at 12 and 36 hours with addition of low fluence UV-B in WT plants (Fig. 7.35). *hy5* mutants had significantly higher metallochaperone 2 transcript at 12 and 36 hours with and without UV-B as compared to WT. *rup1* mutants had no significant change in metallochaperone 2 transcript at 12 and 36 hours without UV-B but significantly lower levels at 12 and 36 hours with the addition of UV-B as compared to WT. UVR8 OE plants had significantly lower metallochaperone 2 transcript at all time points and UV-B conditions except at 36 hours without UV-B were a lower but non-significant count was observed (Fig. 7.35).

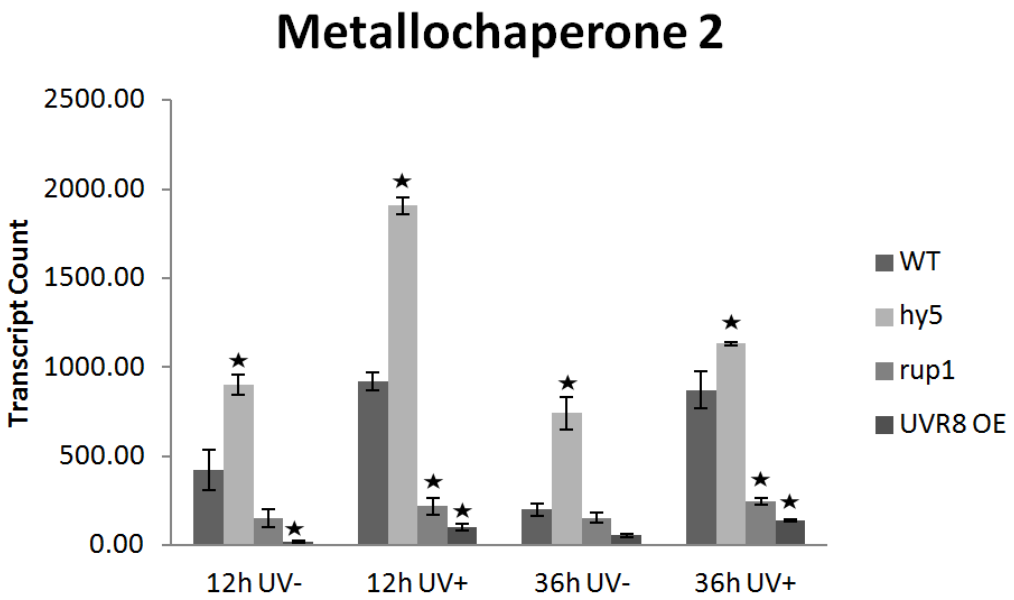



Figure 7.35: Metallochaperone 2 nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). Metallochaperone 2 gene model = Mapoly0180s0021.1

A peroxidase gene showed up-regulation previously under UV-B condition as so was included in nCounter analysis. WT plants showed small increases in peroxidase gene transcript at 12 and 36 hours in response to UV-B (Fig. 7.36). *hy5* mutant plants had a dramatic increase in transcript at 12 hours with addition of UV-B as compared to WT. *rup1* and UVR8 OE plants had dramatically reduced transcript counts as compared to WT at all time points and UV-B conditions. Low transcript counts resulted in no statistically significant differences in transcript counts but overall we see increased transcripts in WT and *hy5* mutant plants, and a lack of transcripts in *rup1* mutant and UVR8 OE plants (Fig. 7.36).

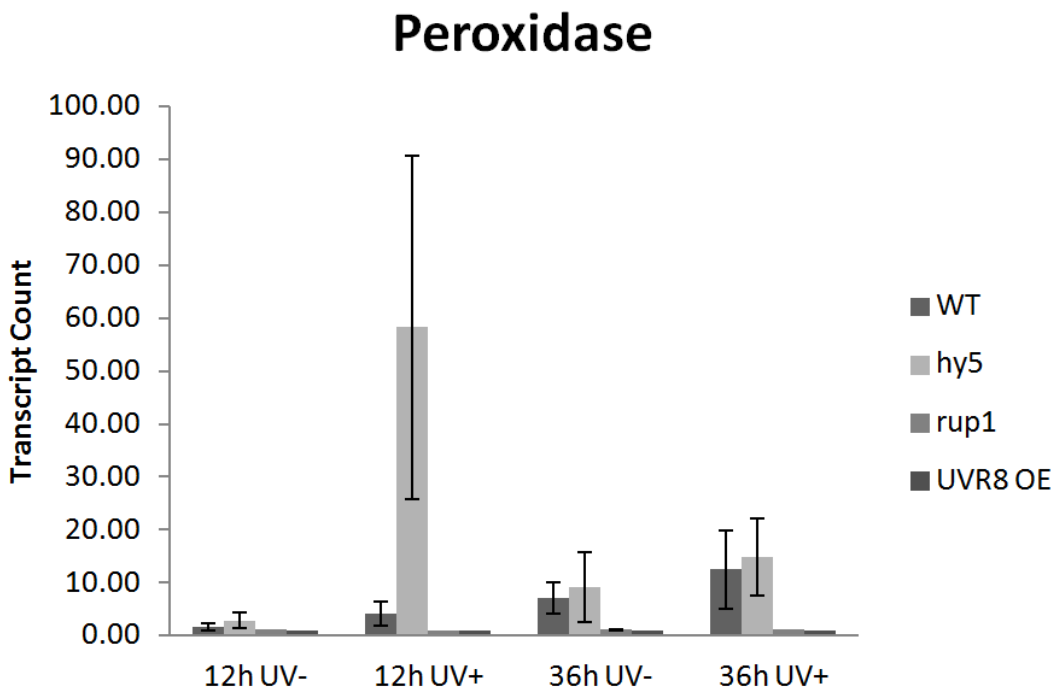



Figure 7.36: Peroxidase nCounter analysis. Transcript count is shown at corresponding time points under UV- and UV+ treatments. WT, *hy5* and *rup1* mutants, and the UVR8 over-expressor transcript counts are each shown. * indicates significant difference from WT ($P < 0.05$). Error bars = standard error (n=3). Peroxidase gene model = Mapoly0106s0052.1

## 7.3 Discussion

*M. polymorpha* has been shown to respond to UV-B through the production of flavonoids, in particular flavones. This production involves likely activation of the UVR8 pathway and flavonoid biosynthetic genes while stress response genes may play alternative roles in the UV-B response. As such we looked at the specific regulation of a core set of genes that are involved in the UV-B response of *M. polymorpha* under low fluence at two separate time points, 12 and 36 hours. These time points were sampled at time points were circadian rhythm affect would have had the least influence, although any circadian rhythm effect was unlikely in this study. These time points were at the end of the first and second day of UV-B treatment and thus would show the highest up-regulation of UV-B specific genes. By using mutants of *hy5* and *rup1* as well as UVR8 OE we were also able to determine how different flavonoid production and subsequently different regulation affected the UV-B response.

### 7.3.1 Analysis of UVR8 pathway expression under UV-B conditions

The UVR8 pathway is responsible for the perception of UV-B and is the source of the signal transduction to activate flavonoid production in response to UV-B. nCounter analysis of the UVR8 gene showed that the regulation in *hy5* and *rup1* mutants had no change in UVR8 transcript while, as expected UVR8 OE plants had dramatic increases in UVR8 transcripts. As HY5 and RUP1 act downstream from UVR8 this result is expected. UVR8 interacts with COP1 upon UV-B perception and we observe that *rup1* and UVR8 OE plants have up-regulation of COP1. UVR8 OE plants have high transcript of UVR8 so the increased COP1 expression in response to UV-B may be due to the increased requirement for interaction with UVR8 as it forms active monomers upon UV-B perception. Increases in COP1 transcript in *rup1* mutants may be due to the loss of UVR8 re-dimerisation capability and subsequently UVR8 remains in its active form which would require higher COP1 interaction and thus increase transcription. However *rup1* mutants at 12 hours still contain transcripts for RUP1. The activity of protein made from these transcripts is unknown although the mutation corresponds to a 53 bp deletion (Nick Albert, personal communication, 2017) which likely causes no active protein to form. This is somewhat supported by the gene regulation pattern in other responsive genes of *rup1* mutants at 12 hours that do not match WT plant gene transcript patterns and more closely matches the 36 hour sample which contains reduced RUP1 transcript. Nevertheless caution must be used when analysing the results of the *rup1* mutant at 12 hours. We also see that HY5 transcripts are increased in the *rup1* and UVR8 OE plants as the UVR8:COP1 interaction activates the downstream pathway. *hy5* mutant plants understandably had low levels of HY5 transcript.

HY5 is a major regulator of the UV-B pathway and as such its own regulation is important in the UV-B response. Previously it has been shown that SUB1, which is a negative regulator of HY5, is involved in the UV-B response (Guo et al., 2001). In *hy5* mutants we see that HY5 is decreased and SUB1 transcripts increased, further linking these two factors. Alternately we see that as HY5 increases in the *rup1* mutant and UVR8 OE plants, SUB1 transcript decreases. SUB1 and HY5 represent a dynamic point of cross talk between UV-B, cryptochrome and phytochrome pathways and may be responsible for balancing the response largely through HY5 levels. HY5 has also been linked to ELIP2 for the protection of plants in response to UV-B (Hayami et al., 2015). HY5 may interact with ELIP2 directly and we see that in *hy5* mutants

ELIP2 is also severely reduced. This reduction in ELIP levels may also contribute to the enhanced damage we see in *hy5* plants upon UV-B exposure at high fluence (Chapter 6). ELIP2 levels are significantly higher in UVR8 OE plants at 12 hours with UV-B, and this also corresponds with an increase in HY5 transcript further supporting the link between HY5 and ELIP2 and consequently protection from UV-B.

SPA which in association with COP1 is responsible for degradation of HY5 in the dark showed no change in transcript count from WT plants in any of the plant lines tested. As all sampling times were conducted in the light period and at the end of a UV-B exposure it was assumed that regulation of SPA would be unaffected.

### 7.3.2 Analysis of putative flavonoid transcription factors

A range of putative transcription factors were found that may be involved in the flavonoid pathway in response to UV-B. MYB14 had previously been analysed and found to increase in response to UV-B and result in increased flavone levels (Chapter 6). MYB14 was also associated with the stress response pathway rather than direct activation due to UVR8 signalling. In *hy5* mutant plants we see that MYB14 expression increases yet flavonoids are severely reduced in *hy5* plants. This increased expression may be due to stress related signalling rather than direct UV-B signalling through UVR8. *hy5* mutants also show susceptibility to UV-B and may strongly induce stress related genes upon UV-B irradiance. This activation may induce MYB14 for the production of protective secondary metabolites, independent of UVR8, such as flavones, riccionidinA or other secondary metabolites. *rup1* mutant and UVR8 OE plants that had increased flavones and elevated protection to UV-B, show a decrease in MYB14 expression upon UV-B irradiance as compared to wild type. This reduction may be due to the increased protection of flavonoids which results in a reduced stress response and therefore reduced induction of stress related MYB14. The response of MYB14 is further linked to stress related pathways by its activation in nutrient stress (Albert et al., 2018). MYB2 may function in a similar stress related manner, as we see reduction in transcript in the *rup1* mutant and UVR8 OE plants, similar to that of MYB14.

GL3 had previously been found to increase in response to high and low fluence UV-B at 1 day (Chapter 5). In the low fluence treatment used here at 12 and 36 hours we see little up-

regulation in WT plants (Fig. 7.11). *hy5* plants show no significant change in transcript count from WT plants yet *rup1* mutant and UVR8 OE plants are significantly reduced in GL3 transcript (Fig. 7.11). The previous data supported GL3 as being indirect of the UV-B pathway and involved in the stress response, which these results support. Similar to MYB14 we see reduced transcript in those plants with the greatest protection against UV-B and subsequently lowest assumed stress response. This pattern is also consistent with another putative transcription factor, VQ motif. This was previously described as a WRKY related protein (Chapter 5), and involved in both abiotic and biotic responses. nCounter analysis shows the same trend as MYB14 and GL3 (Fig. 7.10) and we may tentatively describe this factor as involved in the indirect response for the reduction of stress upon UV-B irradiance. The question arises as to why so many identified putative transcription factors seem to be involved in the stress response rather than direct UV-B response. Sampling time for RNA-seq may largely explain this as low fluence and high fluence sampling involved 1 day samples which would have indirectly favoured genes that may be activated later in the UV-B response for stress mitigation. The early UV-B time point on the other hand at 4 hours may not have captured all the transcription factors that are highly induced directly through UV-B mediated signalling or their up-regulation may not have been high enough to be picked up in data sets at this early time point. Future work could use a time point of 12 hours similar to that used in nCounter study to attempt to capture more genes directly activated by UV-B alone.

### 7.3.3 Analysis of flavonoid biosynthetic genes

Two PAL candidates were analysed by nCounter, PAL3 and PAL4. PAL3 was found to have increased transcript in *hy5* mutants and a reduction in transcript in UVR8 OE plants (Fig. 7.13). PAL4 contrastingly had reduced *hy5* transcripts and increased transcript in *rup1* mutant and UVR8 OE plants in response to UV-B (Fig. 7.14). PAL catalyses the first committed step in the production of phenylproponoids and therefore represents an important decision point in the production of flavonoids in response to UV-B. The response of PAL4 in *hy5* mutants represents an expected result as the reduced response through UVR8, likely due to reduced HY5, results in a reduced response in PAL4 and subsequently lower flavonoid production. Conversely the increased response through UVR8 by *rup1*

mutant and UVR8 OE plants results in an increased response in PAL4 transcript and subsequently higher flavonoid production. However, PAL3 exhibits the opposite effect with high transcript in the *hy5* mutant and low transcript in the *rup1* mutant and UVR8 OE plants. This contrast may be due to alternate function or regulation of the different PAL genes in *M. polymorpha*. PAL may be regulated by multiple factors including the MYB transcription factors (Zhang & Liu, 2015). This leads to a hypothesis that PAL3 and 4 are regulated differently by separate MYB, or other, transcription factors. Potentially PAL3 could be regulated by a MYB such as MYB14 which would be induced upon stress responses while PAL4 is regulated directly through the UVR8 pathway by a separate as yet unidentified MYB. This would explain the difference in expression pattern we observe between PAL3 and 4. The expression pattern of MYB14 and PAL3 may fit this model as we observe increased MYB14 expression in *hy5* mutant plants and subsequently increased PAL3 expression. Conversely we observe reduced MYB14 expression in *rup1* mutants and UVR8 OE plants and reduced PAL3 expression. MYB14 OE plants do not only increase flavone compounds but also the compound riccionidinA (Albert and Davies Unpublished). However, whether the up-regulation of PAL3 contributes to the production of other compounds such as riccionidinA is unknown. This alternate expression from PAL3 would be interesting in that it could lead to formation of secondary metabolite required for stress responses rather than UV-B responsive flavonoids alone. The interaction partners of MYB14 are unknown in *M. polymorpha* and will be an interesting point for further study.

Two CHS candidates were also analysed using nCounter with low fluence UV-B. CHS 21-159 was expressed under UV-B at 12 and 36 hours (Fig. 7.16), while CHS 70-36 only increased in expression at 36 hours with UV-B in WT plants (Fig. 7.17). This leads to the hypothesis that CHS 21-159 is expressed in a UV-B specific manner through the UVR8 pathway while CHS 70-36 may be expressed independently of UV-B and likely in a stress related manner. CHS 70-36 expression at later time points where stress may occur is indicative of this, and aligns with previously identified high expression under high fluence treatment at 1 day (Chapter 5). Analysis in *hy5* mutant plants showed that CHS 21-159 transcript was significantly reduced as compared to WT, while *rup1* and in particular UVR8 OE plants had enhanced CHS 21-159 transcript in response to UV-B. Together this indicates that CHS 21-159 is directly regulated through the UVR8 pathway, likely in a HY5 dependent manner. CHS 70-36 however, was not affected in *hy5* mutant plants but showed reduced transcript in *rup1* and UVR8 OE plants.

This indicated the protection in flavonoid over-expressing plants that may reduce the stress response, may also reduce CHS 70-36 transcript and provides a link between stress response and this CHS gene. CHS 70-36 also aligns closely with a stilbenecarboxylate synthase 1 gene based on nucleotide identity. Stilbenes have been found to be induced upon pathogen attack and even postulated to have a protective measure against UV through the reduction of oxidative stress (He *et al.*, 2008, Chong *et al.*, 2009). Whether CHS 70-36 functions in this way is unknown, but its increased expression under high fluence UV-B, later time points and reduced expression in flavonoid protected plants certainly suggests a protective function linked with indirect effects of UV-B such as ROS.

We observe that two different candidate genes for PAL and CHS responded differently under the UV-B conditions used in our nCounter study. Analysis of differential transcript counts in the mutant and over expression plants, which have altered flavonoid amounts, reveals that they may function in both direct and indirect pathways. The indirect pathway in *M. polymorpha*, which may be largely induced by stress related factors, may respond first through MYB14 which could activate PAL3 and CHS70-36 for the production of flavones but also other secondary compounds such as riccionidinA and stilbenes. In contrast the direct UVR8 pathway in *M. polymorpha* responds through HY5 with activation of PAL4 and CHS21-159, similar to the response seen in higher plants. This results in increased flavonoid compounds for the mitigation of UV-B effects through ROS scavenging or UV-B absorbing flavones. It must be noted these differences would not be fully independent and PAL4 activation for flavonoid production is highly likely to also occur under stress conditions. This hypothesis would need to be further studied to determine the transcription factors and partners that could allow for a possible differential responses.

A CHI and CHI-L candidate were each also analysed and showed differential expression in the tested plant lines. CHI was seen to be unaffected by the loss of HY5 in *hy5* plants while its transcript counts increased in *rup1* and UVR8 OE plants in response to UV-B. CHI may therefore be regulated independently of HY5 but still in a UVR8 dependent manner in response to UV-B. CHI-L which has an enhancer function had significantly lower transcript in *hy5* mutants indicating that HY5 may act to enhance CHI-L activity. *rup1* mutant and UVR8 OE plant had increased activity of CHI-L under UV-B conditions which may be due to the enhanced UVR8 pathway in these plants, and subsequently higher HY5, acting on CHI-L.

Overall we see that CHI and CHI-L genes in *M. polymorpha* function in a UV-B dependent manner to increase flavonoid biosynthesis.

Two DOX candidates were also analysed which may function as the FNS and FNR genes in *M. polymorpha*. Assignment of each is difficult and would require further analysis but we can see from previous data and nCounter analysis that both are regulated in a UV-B dependent manner. The *hy5* mutant that has a reduction in flavonoids also has a reduction in expression of both DOX 28 and 38 in response to UV-B. *rup1* mutant and UVR8 OE plants which have enhanced flavonoid amounts both have increased expression of both DOX 28 and 38 in response to UV-B and also in UV-B lacking plants (Fig. 7.20, 7.21). While the exact function of these genes as FNS or FNR is unknown, it is further evidence that they both function to enhance flavonoids in *M. polymorpha* in response to UV-B.

### 7.3.4 Analysis of putative stress and indirect pathway genes in response to UV-B

A number of other genes that were found to respond to UV-B but not directly involved in flavonoid synthesis were also analysed in our nCounter data set. These genes largely indicated the stress response of *M. polymorpha* in response to UV-B and provide an understanding of how the manipulation of the flavonoid profile affects the UV-B response. Overall we summarise each mutant and over-expressor response from UVR8 perception through to the putative effects on photosynthetic machinery (Fig. 7.36, 7.37, 7.38).

*hy5* mutant plants, which have reduced flavonoids, were shown to have reduced expression of ELIP, CAB, RUBISCOssu genes as compared to WT in response to UV-B. *hy5* mutant plants also had increased expression of peroxidase, metallochaperone and ERF genes as compared to WT in response to UV-B. The reduction in ELIP has previously been described and may be linked with the direct reduction of HY5 in *hy5* mutants. Two of three CAB genes analysed showed reduced expression in response to UV-B at 36 hours while a third, CAB68-87, had enhanced expression. The reduction in CAB genes may reflect a loss in chlorophyll in *hy5* mutant plants which may explain the browning affect that is apparent in *hy5* mutants in response to UV-B (Chapter 6). CAB68-87 expression pattern is harder to interpret but may indicate an increased expression in response to stress in order to protect the photosynthetic machinery in *hy5* plants. However, we also see high expression in *rup1* mutants and no

expression in UVR8 OE plants which suggest the function may be independent of the stress response. CAB68-87 is a putative assignment and the study of its specific functions in relation to UV-B or general stress would be an interesting point of future study. *Hy5* plants also show a drop in RUBISCOssu transcript, which also may indicate damage to the photosynthetic machinery from UV-B. ERF transcripts are also up-regulated in *hy5* mutants similar to that of WT and indicate a role for ethylene in the response of *M. polymorpha* to UV-B. A peroxidase gene is also shown to be up-regulated in response to UV-B as compared to WT in the *hy5* mutant, especially at 12 hours. This up-regulation may indicate that the ROS response in *hy5* mutants is higher than in WT. Previously we have shown that the lack of flavonoids results in an increase in ROS in *M. polymorpha* (Chapter 6) so the up-regulation of a peroxidase gene in *hy5* mutants, which have reduced flavone content, is not unexpected. Metallochaperone genes which were putatively assigned as functioning in *M. polymorpha* to help protect the chloroplast through reduction in ROS in response to UV-B, are also up-regulated in the *hy5* mutants as compared to WT. This up-regulation when linked with peroxidase up-regulation may further indicate that *hy5* plants undergo higher ROS burden in response to UV-B. This increased ROS burden may likely be the result of the reduction of protective UV-B absorbing or ROS scavenging flavonoids in *hy5* plants. In the general stress response we see that the DNA repair genes analysed are largely unaffected in the *hy5* mutant while HSP's show both increased and reduced expression in response to UV-B as compared to WT. It may be that at the low fluence use in our nCounter analysis we do not see that same high response in the DNA and HSP genes that we had previously seen in high fluence analysis. DNA damage may be similar to WT, as *hy5* mutant plants, while having reduced flavonoids, contain small amounts of flavonoids which may be sufficient for protection of DNA. Alternatively these small flavonoid amounts may localise to the nucleus for DNA protection as has been described in other higher plants (Agati et al., 2012). Further study on the flavonoid organelle localisation in *M. polymorpha* would provide further understanding of their exact function, even at low levels such as in the *hy5* mutant plants. Overall we see that the down-regulation of the flavonoid pathway due to the loss of HY5 results in an increased stress related response indicating that UV-B has a stronger negative influence, likely due to higher ROS, in these plants (Fig. 7.37).

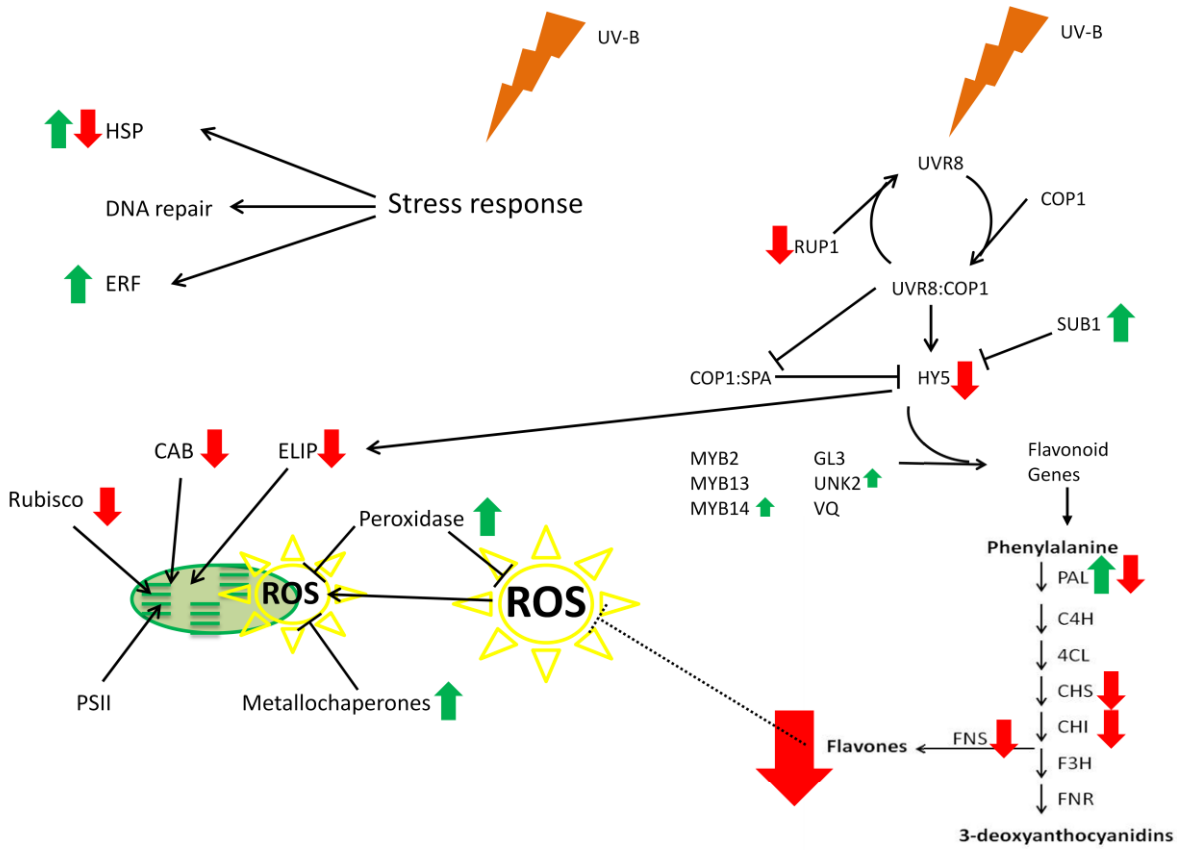



Figure 7.37: Proposed model of UV-B gene response in *hy5* mutant *M. polymorpha*. Green arrows indicate up-regulation of putative genes while red arrows indicate down-regulation.

*rup1* mutant and UVR8 OE plants have elevated amounts of flavones and we hypothesis that this would give an increased protection against UV-B, in contrast to that of *hy5* mutants. Subsequently we see that the peroxidase and metallochaperone genes have significantly lower transcript counts which may indicate that lower ROS burden occurs in these plants as compared to WT and *hy5* mutants. As previously suggested the increased flavone content in these plants may be responsible for a decrease in ROS. ELIP2 transcripts are seen to increase in UVR8 OE plants but this may be due to higher HY5 transcript rather than increased transcript for the protection of photosynthetic machinery. PSII transcript was seen to decrease in both *rup1* and UVR8 OE plants. This gene is responsible for PSII D1 protein synthesis and may indicate a reduced need for D1 turnover in these plants as the PSII system is protected at a greater level from UV-B related damage in these plants as compared to WT and *hy5* mutants. HSP, DNA repair and ERF transcript counts were all lower than WT plants and also indicate that the overall stress response in these plants is lower than in WT. This

reduction in perceived stress in these plants may be hypothesised to be due to the increased flavonoid production within these plants reducing both ROS and incident UV-B damaging affects in the cell.

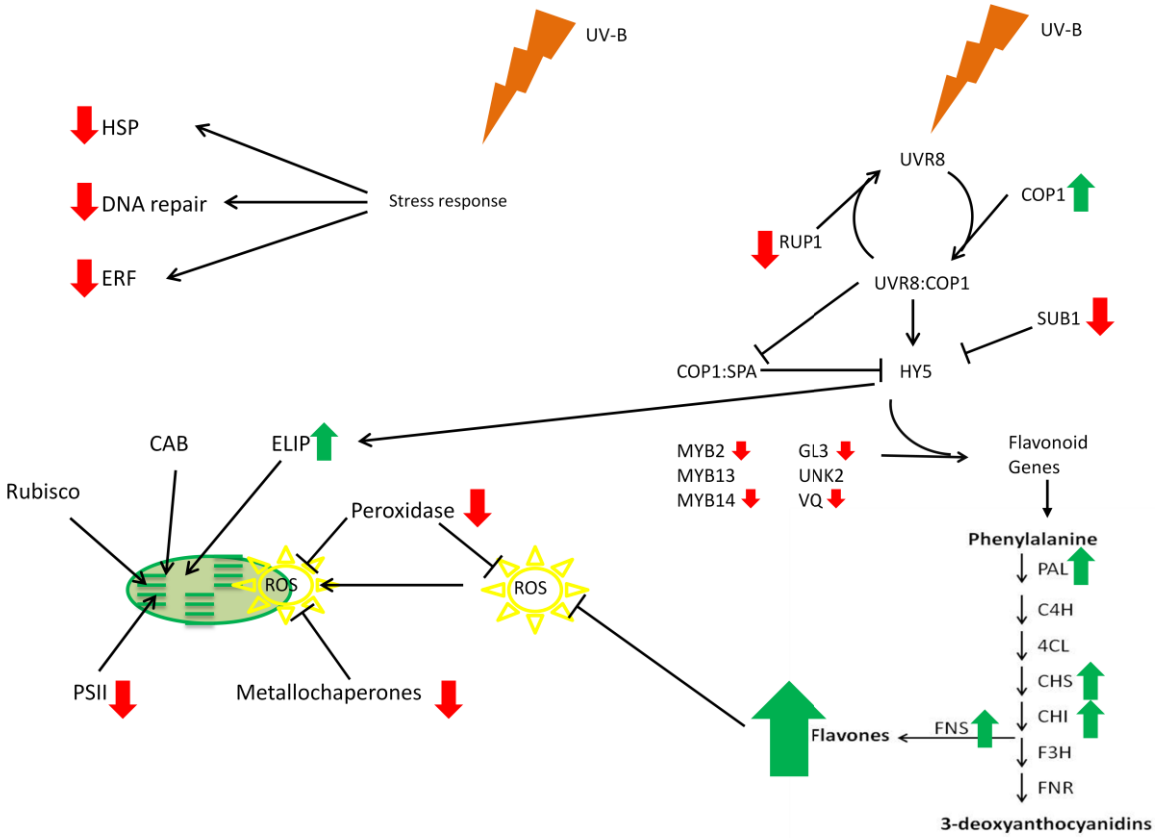



Figure 7.38: Proposed model of UV-B gene response in *rup1* mutant *M. polymorpha*. Green arrows indicate up-regulation of putative genes while red arrows indicate down-regulation.

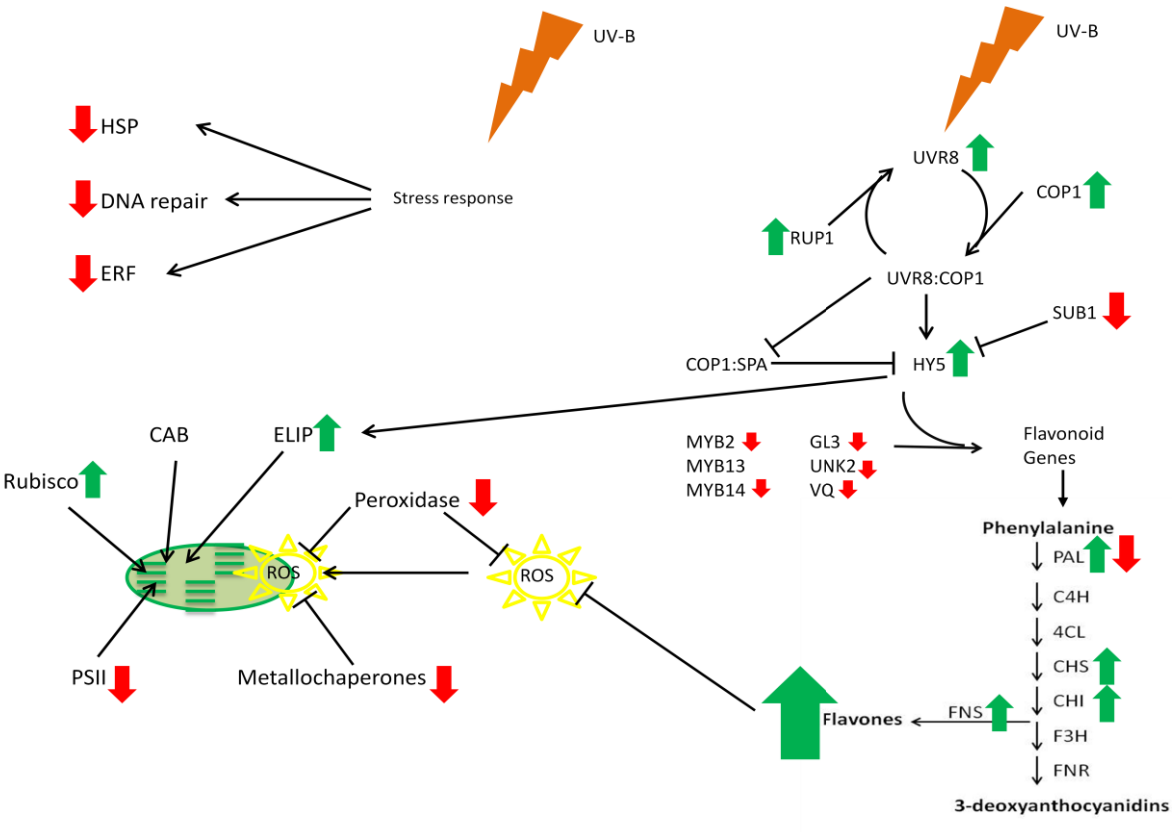



Figure 7.39: Proposed model of UV-B gene response in UVR8 over-expressor *M. polymorpha*. Green arrows indicate up-regulation of putative genes while red arrows indicate down-regulation.

# Chapter 8: General discussion

The overall aim of this thesis was to determine if *M. polymorpha* responded to UV-B through the production of flavonoid compounds, whether these compounds were required for UV-B tolerance and the genetic elements that were required for the response. It was shown that *M. polymorpha* produces flavones and reduction in flavone content resulted in increased susceptibility to UV-B. *M. polymorpha* was also shown to respond through the specific UVR8 pathway and also through non-specific UV-B pathways for protection.

In particular low fluence UV-B irradiation is able to increase flavone content in thallus tissue dramatically. This low fluence UV-B irradiation level is similar to levels commonly found within the light environment liverworts would be expected to grow under. Such increase in response to UV-B showed that the mechanism for flavone production may depend largely on a UV-B mediated pathway, most likely through UVR8 activation of flavonoid biosynthetic genes. Additionally, high fluence UV-B was able to exhibit a similar response through the production of flavone compounds, yet some key differences were observed between the two treatments. High fluence in particular resulted in increased production of luteolin-based flavones while low fluence treatment resulted in higher levels of the apigenin flavones. This difference may be largely due to the function each flavone may play within the cell. Apigenin may be largely a UV-B screening compound while luteolin may function to scavenge ROS. These results were in contrast to a previous study where *M. polymorpha* was shown not to increase flavonoid levels but change the ratio of apigenin to luteolin in favour of luteolin (Markham et al., 1998a). However, the methodology used differed greatly in our study and may reflect the different results obtained. Combining both results we can conclude that *M. polymorpha* responds to UV-B through the production of flavone compounds and the ratio of apigenin to luteolin may also be an important factor in mediating the response through UV-B absorbing or ROS scavenging functions. Future work on how the ratio may change in response to different UV-B or stress events would be particularly interesting. In particular the discovery of a flavonoid 3-hydroxylase (F3H) that could alter the ratio between apigenin and luteolin would be interesting and would indicate secondary control against different UV-B fluence independent of UVR8 sensing. The regulation of such a gene in response to high

and low fluence and the related change in apigenin to luteolin ratio could give a better understanding of the UV-B protective response in early land plants.

The localisation of flavones gave further clues to each function as low fluence plants that were enhanced for apigenin, had high levels of epidermal flavonoids while the high fluence treated plants had lower accumulation in the epidermal layers. However we were unable to determine the localisation of specific flavonoids compounds in the tissue or localisation in cell compartments. Future work to determine the composition of cellular flavonoids in comparison to the epidermal flavonoids may lead to a better understanding of the function of each in these early land plants. Further analysis using confocal microscopy to look at the cellular content may also provide an understanding of flavonoid function in *M. polymorpha*. In addition, the use of new techniques such as Raman spectroscopy could analyse specific regions such as the epidermis and give insight into the localisation of flavone compounds to help provide insight for their function (Gierlinger *et al.*, 2012, Mateu *et al.*, 2016). In thallus tissue we observed high levels of ROS in high fluence treated plants within the chloroplast and the localisation of flavones in these tissues in response to ROS accumulation would be particularly important in order to reduce damage to the delicate photosynthetic machinery. However the high fluence used in this experiment is extremely high when related to the relatively low PAR level we used. Such conditions are very unlikely in the natural environment. The high fluence treatment was useful however to understand the protective ability of plants with enhanced or reduced flavone levels as it gave a distinct UV-B damage phenotype.

The response through flavone compounds indicated involvement of the flavonoid biosynthetic pathway which is known to be mediated in a UVR8 dependent manner under UV-B irradiance. As such, to determine the gene regulation in response to UV-B, plants were used for RNA sequencing and the gene changes analysed. Importantly, we saw up-regulation of the known components that regulate the UVR8 pathway such as COP1, HY5 and RUP1. We also saw a corresponding increase in the activity of flavonoid biosynthetic candidate genes such as PAL, CHS, CHI, CHI-L, FNS and FNR. This matched the production of flavones in response to UV-B and showed that, much like in higher plants, *M. polymorpha* responds through the UVR8 UV-B dependent pathway.

Gene regulation was not confined to changes in the UVR8 and flavonoid pathway however, and we observed large changes in a number of putative genes. Many of these related to an indication of enhanced stress response from the plant. This was expected as UV-B can act through its absorption by many different components in the cell such as DNA, proteins and lipids in cell membranes and specific organelles, such as chloroplasts. As such we saw increases in genes relating to the reduction of ROS, DNA repair and chaperones. These all indicated a generic stress response upon UV-B irradiation indicating the flavonoids alone are not sufficient to deal with the effects of UV-B and rather a whole plant response must be required.

Of particular interest was the regulation of HY5 and the genes that interact with HY5. HY5 is a key regulator of not just the UV-B response but also the blue light and far-red response mediated through cryptochrome and phytochrome. As such its regulation is an important point of cross-talk in the light response of plants. RNA-seq showed HY5 was highly up-regulated in response to UV-B. Mutation to reduce HY5 did not result in a loss of flavone production but rather an inability of plants to respond to UV-B through increases in flavones. This indicated that HY5 is a regulator of the UVR8 mediated activation of the flavone pathway in order to increase flavones in specific response to UV-B in *M. polymorpha*. Conversely, it also indicated that other factors apart from HY5 interact with the biosynthetic pathway for the production of flavonoids. *hy5* mutant plants previously grown in UV-B lacking environments showed increased damage when challenged with both high and low fluence UV-B suggesting that the increased production of flavones is required for UV-B defence in *M. polymorpha*. However plants grown from gemmae under continuous low fluence treatment were able to survive in a stunted form indicating that flavone production via other regulators are sufficient for limited plant survival.

While the response of *M. polymorpha* to UV-B requires HY5 the ability of plants to survive without flavones was also tested through analysis of *chi* mutants. These *chi* mutants were deficient in detectable flavones compounds yet were able to grow without phenotypic difference to WT plants. However, they also exhibited increased damage under challenge with high and low UV-B fluence and the acclimatisation test of gemmae grown under continuous low fluence treatment resulted in severe stunting. This showed flavones are required for plant defence against UV-B in *M. polymorpha*. A CHI-L gene was not required for

flavone production alone but may act to enhance CHI activity as CHI-L mutants had reduced flavone levels. Overall, mutants that displayed a reduction in flavones as compared to WT were hypersensitive to UV-B.

While the reduction of flavone compounds leads to a reduction in plant defence against UV-B the increased production of flavone compounds leads to an enhanced protection against UV-B. This was shown through the mutation of RUP1 and the over-expression of a MYB transcription factor MYB14. RUP1 regulates the recycling of UVR8 from its active form back to its inactive form and as such mutation to reduce RUP1 would result in higher amounts of constitutively active UVR8. The function of MYB14 is still largely unknown but may be involved in the flavonoid pathway and in particular the production of riccionidinA in response to stress (Albert and Davies Unpublished). Both *rup1* and 35s:MYB14 plants had enhanced levels of flavones and both showed enhanced protection against UV-B, in particular to challenge by high fluence treatments. However *rup1* mutants were severely stunted under UV-B acclimation. This stunting may be due to over activation of the UVR8 pathway and flavonoid production during key developmental stages as flavonoid compounds are required not just for UV-B protection but other growth related functions such as auxin transport.

Overall we observed that those plants that had reduced or lacked flavone compounds were hypersensitive to UV-B, while those that had enhanced levels of flavones had enhanced protection against UV-B. This indicated that flavones in *M. polymorpha* are required for the protection against UV-B.

Further analyses of mutant and over-expression lines by nCounter reveal the regulation of key genes in response to UV-B. In particular it was identified that both direct and indirect pathways may act for the production of secondary metabolites, not limited to flavones, in order to protect plants against UV-B. In particular different PAL and CHS genes may be regulated either in response to stress factors or directly through the UVR8 pathway. This differential expression would allow *M. polymorpha* to respond to broad ranges of stress through the production of flavonoids, not just to UV-B stress alone. Related to early land plants, of which *M. polymorpha* may be the closest representation, we can see that they likely had the genetic machinery capable of dealing with UV-B and generic plant stress when

making the transition onto land environments. While we observed that stress and UV-B related factors were involved in the response of *M. polymorpha* the interaction of putative transcription factors is still unknown. We postulated that HY5, as in higher plants, may be a key mediator of the UVR8 pathway while other factors such as MYB14, a VQ motif protein or GL3 may be involved in the stress related response. Future work around the interaction of these partners would be particularly interesting to determine the regulation required for the differential response we observe in this study.

*M. polymorpha* as a closest representation to that of the early land plants appears to respond through a conserved pathway also utilised in higher plants. Genes candidates for the UVR8 pathway such as COP1, HY5 and RUP1 as well as biosynthetic genes for flavone production such as PAL, CHS, CHI and FNS all appear to be present. *M. polymorpha* which belongs to the liverworts are therefore able to produce flavonoids while proposed ancestor green algae species are found to lack flavonoids (Iwashina, 2000, Tohge *et al.*, 2013). This acquisition of flavonoid compounds in liverworts as they appeared on land has been proposed to have occurred in order to help defend against the UV-B environment these plants now faced (Iwashina, 2000). Correspondingly we see that *M. polymorpha* indeed responds with an increase in flavonoids resulting in protection while loss of flavonoids results in susceptibility to UV-B. This further strengthen the hypothesis that the acquisition of flavonoid biosynthesis in the early land plants provided protection against UV-B and helped facilitate the colonisation of land. While liverworts are proposed to be the first extant relatives of the early land plants they may not be the least evolved plants on land today. In fact liverworts have had the longest time on land to go through adaptive change and may not represent the exact plants that faced the challenges of moving from aquatic environments onto land. As such the gene regulation we see in these plants may not be viewed as a snapshot in time to early land plants but rather an analysis of how the early land plants may have evolved to function in today's environments. However these liverworts still give the best indication of the response of the early land plants to environmental stresses such as UV-B.

In conclusion this research confirms the hypothesis that flavonoids are produced in response to UV-B, in particular flavones, and that these flavonoids are required for the protection against UV-B. Production of flavonoids in *M. polymorpha* is also largely mediated through the conserved UVR8 pathway.

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# Appendices

**Appendix 1: Table of primers used for qRT-PCR analysis**

| Primer | Forward 5'-3' | Reverse 5'-3' |
|---|---|---|
| Actin | GATCTTGCTGGACGTGATCTT | GCTTCTCCTTCATGTCTCTCAC |
| CHS | CACCAACAGCAATGATAAGC | GTCTACCCCACTCTTTGATG |

**Appendix 2: RNA-seq analysis**

Refer to supplied files

**Appendix 3: Ncounter results**

Refer to supplied files